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INSIGHT INTO EYESIGHT THROUGH RESONANCE RAMAN

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ONE way to appreciate the wondrous sense of normal human eyesight is to imagine an attempt to duplicate it. We would begin with an elaborate set of fore optics: a lens fully corrected for all geometric aberrations, automatically and almost instantaneously focusable from a few centimeters out to infinity; an iris diaphragm with automatic adjustment to varying light intensities; a servo-controlled, two-axis scanning mechanism; and, as an elegant touch, a window-washing apparatus capable of all-weather operation with never a need for a refill or defroster.

To two sets (for stereoscopic vision) of such fore optics we would attach a pair of color TV cameras. Their performance would surpass that presently available in at least two respects. Framing rate would be sped up from 1/30 sec to milliseconds. Responsivity would be improved to approach the quantum efficiency, near unity, of the dark-adapted human eye; furthermore, this remarkable ability to detect a single photon is required at every wavelength in the visible region. Finally, we would devise the circuitry necessary to integrate the sensed signals to a form acceptable to a central processing unit (CPU).

The two sets of simultaneous data continuously received by the CPU would then be ready to be unscrambled. Provided every subsystem were in order, we might wind up with the equivalent of human vision: a continuous, real-time, stereo, full-color display of moving scenes varying in intensity from starlight to sunlight.

Here at Cornell we have concentrated our efforts on trying to untangle only one phase of the complex process of vision, the transduction of photons of light energy into neural responses perceivable (after appropriate integration) by the brain, the biological version of the CPU.

The process of visual transduction is initiated when light is focused by the lens (see Fig 1) onto the retina, a thin layer of tissue lining the back of the eyeball. Enmeshed in this tissue are two types of photoreceptor, or light transducing, cells: rods and cones. The rods are responsible for black-and-white and low light level vision; they are present in the eyes of all sighted animals, sensitizing the entire retina. On the other hand, in primates cones are concentrated in only one small area of the retina, the fovea. So far our experiments have been limited to the rods.

Fig 2 depicts a cross section of the retina. Entering from the bottom, light pours through a series of neuronal layers. Their position notwithstanding, these layers do not take part in the light absorption which is the first step in the visual process but rather serve as an interface between the sensors (both rods and cones) and the optic nerve. Assembling and integrating the information from all of the cells, they transmit it in a sequence and form which is interpretable by the brain. Beyond the photoreceptor cells is the pigment epithelium. One of the roles of this layer of tissue is apparently chemical regeneration. The chemical changes that cause vision must finally be reversed to reactivate the sensors; pigment epithelium functions as a recycling bath where this regeneration takes place [1]. It also has other important functions, such as decreasing scattering in the eye, especially at high light levels. The lack of pigment in the epithelium accounts for the poor sight of albinos under such conditions.

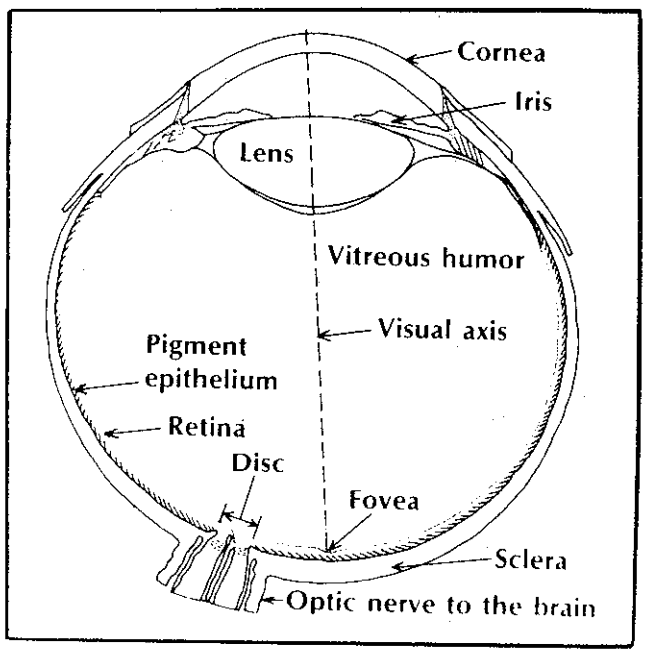

Fig 1 Cross section of a primate eye. Entering light is focused by the lens then passes through a clear, jelly-like substance (vitreous humor) onto the retina, a thin strip of tissue lining the back of the eyeball. The retina contains cone and rod photoreceptor cells. The cone cells, responsible for color vision, are centered in the fovea, a small identation in the retina; the rod cells, responsible for black-and-white and dim light vision, are distributed throughout the rest of the retina.

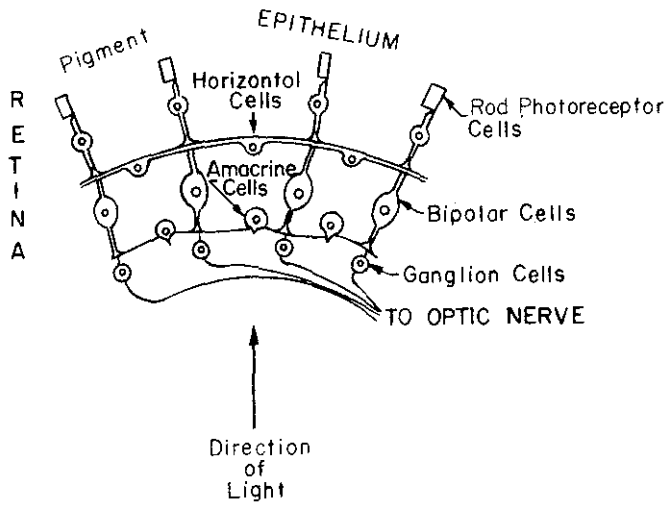

Fig 2 Cross section of the retina. After passing through a neuronal layer (ganglion cells, bipolar cells, amacrine cells, and horizontal cells) light impinges on the rod photoreceptor cells which absorb the light and generate a neuronal response. Light that passes through the retina finally strikes a pigmented layer called the pigment epithelium.

Fig 3 is a cross section of a rod cell. Light enters the inner segment through its electrical connector, the synaptic terminal. The nucleus is, of course, the basic replicating element of the cell, incidental to light transduction but essential to long years of sight. Collectively the mitochondria are the energy source, the battery, of the cell. They manufacture an energy-storing molecular sink called ATP (adenosine triphosphate). Although the production of ATP by the mitochondria is not related to the initial absorption of a photon, ATP may be instrumental at a later stage in the amplification of the energy of a single photon so it can be perceived by the brain. There is presently no concrete evidence to either support or refute this hypothesis, but Hagins and his coworkers have recently shown that the concentration of ATP does decrease after the rods absorb a photon [2]. What is certain is that photoreceptor cells consume large amounts of ATP and that the role of this ATP is not yet understood.

The part of the cell where the photon is absorbed is known as the outer segment. The light energy is absorbed by a molecule called rhodopsin which converts, within milliseconds, the photon's energy into the chemical species responsible for generating a neural response. Several thousand rhodopsin molecules are embedded in the membrane of a few hundred Frisbee-like discs which are stacked within the rod's outer segment. Surrounding the discs is a plasma membrane, and outside this sheath is a medium containing sodium ions. Unexposed to light these Na^+ ions permeate the plasma membrane giving rise to a "dark current" which is interpreted by the brain as darkness. When light strikes the rhodopsin the flow of Na^+ ions is somehow blocked, causing an electrical imbalance (hyperpolarization) across the plasma membrane. The resulting neural response is finally detected by the brain as vision [3].

What is not fully understood is all of the steps between absorption of the photon and generation of the neural response. Despite the fact that a fundamental breakthrough in research in this area occurred over 40 years ago, an accepted theory to identify and explain the entire train of events is yet to be offered.

Harvard was the scene of much of the pioneering work in visual transduction. There George Wald, who had extracted rhodopsin from eyes in the first of many successful experiments designed to elucidate the visual process [4], was able to follow (mainly through absorption spectroscopy) changes in the chemistry of rhodopsin when it absorbed a photon.

Fig 4 summarizes the knowledge gained to date. Rhodopsin (mol. wt. ~ 36 000) is composed of a protein, lipid, and sugar matrix (opsin) which is complexed to a form of vitamin A called retinal. As will be illustrated later, there are many rhodopsins. All the rhodopsins appear to have the same form of vitamin A for the chromophore, but they differ in the composition of opsin. In fact a rhodopsin-like protein has also recently been found in a bacterium, *Halobacterium halobium*, and as far as is known this rhodopsin is a critical element in the bacterium's bioenergetics.

The vitamin A (retinal) component of rhodopsin is the site of photon absorption. The retinal is connected to the opsin through what is called a Schiff base linkage. Here the retinal, which is an aldehyde with a $-\overset{H}{C}=O$ end group, is complexed to the ϵ-amino (NH_2) group of a lysine residue of the protein to form the retinylidene chromophore of rhodopsin [5].

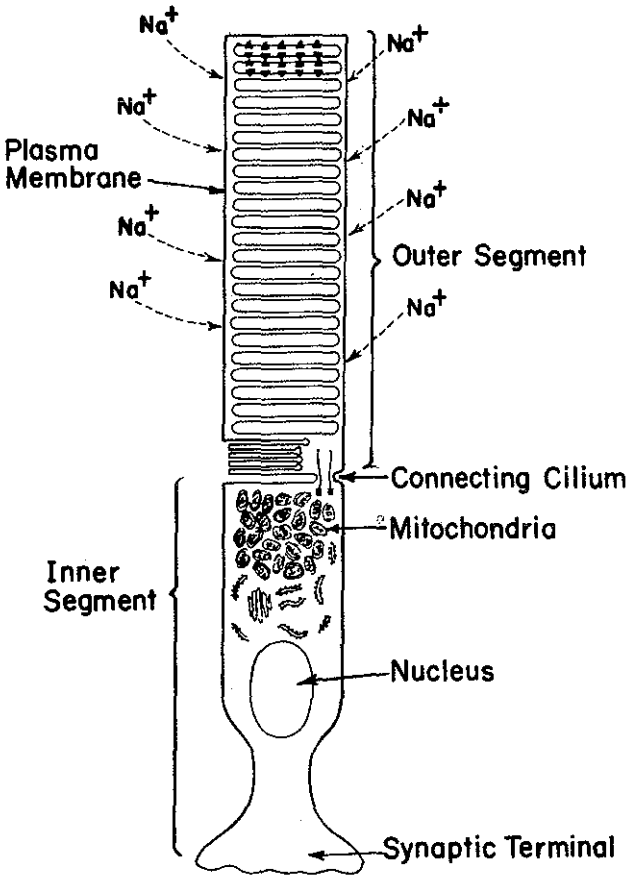

Fig 3 A schematic view of a rod cell in the eye of a vertebrate. The cell has an outer segment containing flat, pancake-like membranous discs in which are embedded the rhodopsin molecules (represented as triangles) in very high concentration. The outer segment is connected to an inner segment containing the nucleus and the mitochondria (the energy source for the cell). The inner segment ends in a synaptic terminal. This connects the photoreceptor cell to the neuronal network which extends out of the retina through the optic nerve and eventually to the brain.

When Wald extracted retinal from its opsin matrix in the dark he found surprising chromatographic and spectroscopic evidence that it was twisted in a constrained *cis* form. (Linus Pauling had felt that this *cis* form was so severly strained as to altogether preclude its existence.) Exposure to light, Wald found, produced the *trans* form. He concluded, quite plausibly, that light triggered the kinked *cis*-retinal back to its unstrained *trans* form. To Wald this isomerization constituted the entire primary photonic event: light was but the catalyst providing the small amount of energy needed to spring the already energized molecule open.

Subsequent work, ours and that of others, has extended that first premise. It is now believed that the isomerization is energized completely by the light, not simply triggered by it [6]. It is known that there are at least five steps in the initial part of the process of vision. In the first step an intermediate species called bathorhodopsin is produced at physiological temperatures in less than 6 psec after the absorption of light by rhodopsin [7, 8], and after a lifetime of about 30 nsec it decays to lumirhodopsin [7].

The transformations of lumirhodopsin to metarhodopsin I and II take place in nanoseconds and milliseconds, respectively, as absorption flash spectroscopy has shown [9]. In vertebrates the neural response begins during the milliseconds between the formation of metarhodopsin I and of metarhodopsin II. Taking minutes, the final change of opsin to free *trans*-retinal is considered ancillary to vision. It appears to be the beginning of the chemical recycling phase. Once a retinal molecule is struck by a photon it is deactivated, and enzymatic reactivation (regeneration) of the *cis* form can be very slow. There are always, however, enough rhodopsin molecules on hand so that vision can proceed without interruption.

Through resonance Raman spectroscopy we have been able to probe the retinylidene chromophore (the center of photochemical activity in vision) by following changes in its vibrational spectrum. Where other forms of spectroscopy — ESR, NMR, circular dichroism, fluorescence, and absorption — have met with at best limited success in further elucidating the process of vision, resonance Raman is proving to be a powerful new tool in the study of the various steps in visual excitation.

Resonance Raman Spectroscopy

Resonance Raman spectroscopy is essentially a specialized branch of Raman spectroscopy, which was discovered independently by C. V. Raman in India and A. Mandelstam in Russia about 50 years ago. Raman spectroscopy is the inelastic scattering of light off the vibrational energy levels of molecules. Classically, to gain a physical appreciation of Raman spectroscopy one considers photons interacting with a vibrating molecule. The photons exchange their momentums with the various vibrational modes, and this causes the molecule to vibrate with larger amplitudes and at higher energy (Stokes scattering). The resulting photons are scattered with less momentum, less energy, lower frequency, and longer wavelength. The differences between the unchanging frequency of the incident photons and the frequencies of the scattered photons are precisely the vibrational frequencies of the molecule under study. Because only one photon in a million is scattered in such an inelastic event the application of the laser, a high-powered, spatially coherent, single-frequency light source, has revolutionized the technique. There are, however, certain major problems with Raman spectroscopy as described above. Firstly, the spectrum one obtains from such an experiment is essentially nonselective. All of the active vibrations of the molecule are observed, and this would result in the spectrum of a 36 000-molecular-weight protein which is quite difficult to interpret. Secondly, Raman scattering even with a laser is still a very weak process and thus insensitive. In essence macromolecules like rhodopsin can only be studied at concentrations of 2 - 3% which is well out of the physiologically relevant concentration range of most proteins and nucleic acids.

Both these problems, selectivity and sensitivity, are overcome in a resonance Raman experiment. In this specialized form of Raman spectroscopy recently developed tunable lasers allow selection of an incident laser frequency which is in resonance (near-coincidence) with the electronic absorption of a particular molecular entity such as the retinylidene chromophore of rhodopsin. In the scattered light the ground state vibrational spectrum of the chromophore (at concentrations of 10^{-5} M or less) is selectively observed above the background of surrounding vibrations from the membrane glycoprotein (opsin) matrix. This selectivity and sensitivity of resonance Raman spectroscopy, along with the advantages of non-resonance Raman — the weak to nonexisting Raman

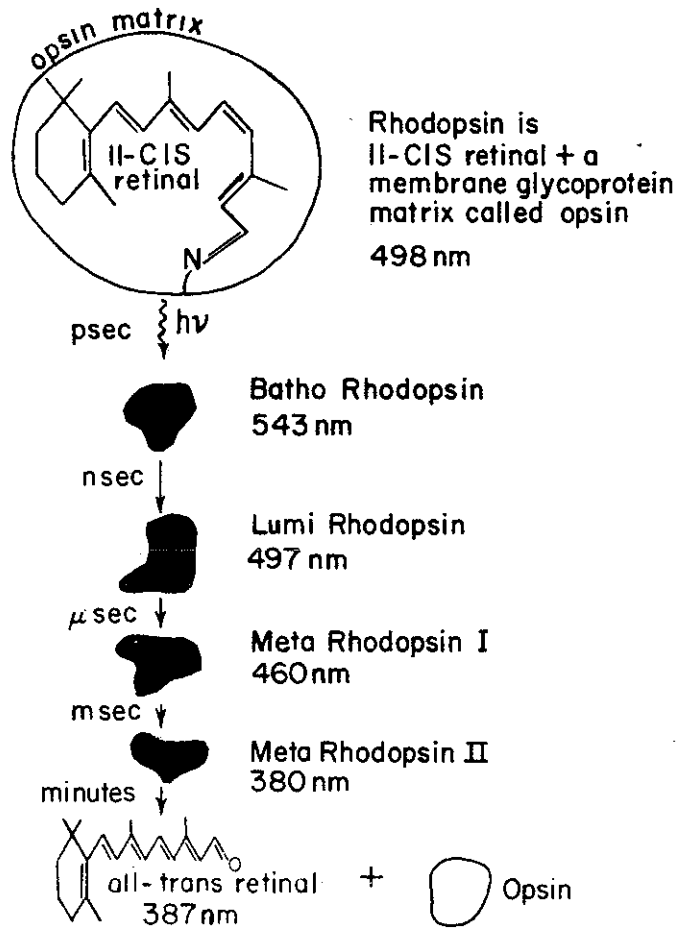

Fig 4 The chemical composition and the light-induced conformational changes of rhodopsin, the principal light absorber in the eye. Rhodopsin is a large molecule consisting of a small (45-atom) unit called retinal embedded in a matrix (of sugar, lipid, and protein) called opsin. The center of photochemical activity is the double-bonded (12-atom) segment of the retinal and those atoms electronically coupled to this region of the molecule. It is the vibration of these atoms that is selectively observed in the resonance Raman spectrum of rhodopsin.

spectrum of water which obliterates ir vibrational spectra in aqueous media and the experimental flexibility of simply scattering a laser beam 1 μm or less in diameter from particular areas of even opaque samples — makes this a new biophysical tool with a great deal of potential.

The serious disadvantage of this technique is fluorescence. Because fluorescence and Raman spectra are often superimposed, and because the intensity of the former is frequently much greater, fluorescence often presents a serious obstacle to Raman studies. Most photobiological systems, however, are found to fluoresce either very weakly or not detectably. Of course this should be expected intuitively: absorbed photons are thereby preserved for photochemistry, not reemitted where they would certainly interfere with vision. Thus, from an experimental standpoint these photobiological systems are ideal for the application of Raman spectroscopy. Furthermore, it has been shown [10, 11] that the ground state vibrational modes that are most intense in resonance Raman are the ones that exhibit large nuclear excursions in the excited state. In summary, for photobiology resonance Raman not only selectively probes the center of photochemical activity in its natural membrane environment, but it also singles out the very vibrational modes that lead the molecule along its photochemical pathway.

Unanswered Questions

The process of visual transduction can actually be subdivided into two related and unanswered questions. First, how is light energy absorbed and stored by rhodopsin? Second, how is the light energy converted into the chemical species which mediates the transport of sodium ions across the plasma membrane to generate the hyperpolarization?

To begin to answer these fundamental questions through resonance Raman spectroscopy we felt that it was important to clarify the chemical constitution of rhodopsin. We obtained the first resonance Raman spectrum of the retinylidene chromophore in an extract of bovine rhodopsin [12, 13]; more recently we have obtained the spectrum with a live eye [14]. By selectively observing the carbon-nitrogen vibration we were able to demonstrate that the Schiff base is protonated, not unprotonated as it is shown in Fig 4. Although this contradicted an earlier suggestion, subsequent workers have also found that the Schiff base linkage is indeed protonated [15, 16, 17]. This observation is significant, for if the linkage were unprotonated it would dramatically alter the photochemical basis of visual transduction. In fact, work in our laboratory has shown that this observation may assume greater importance in the later steps of visual transduction.

Bioenergetics of the Visual Process

The energetics of the interaction of a photon with rhodopsin can be expressed in two divergent ways as depicted in Fig 5. Assuming that the primary photonic event in vision is a simple *cis-trans* conversion, then based on previous investigations of *cis-trans* isomerizations in other systems it would be necessary to assume that bathorhodopsin is at a lower energy than the initial *cis*-rhodopsin (see Fig 5A). The alternative scheme of events seen in Fig 5B, however, appears to us to represent what actually happens in vision. Here part of the energy of the 2-eV photon is absorbed by the *cis*-rhodopsin and is stored in bathorhodopsin. Then visual transduction can really be considered as merely one more example of cellular energy transduction.

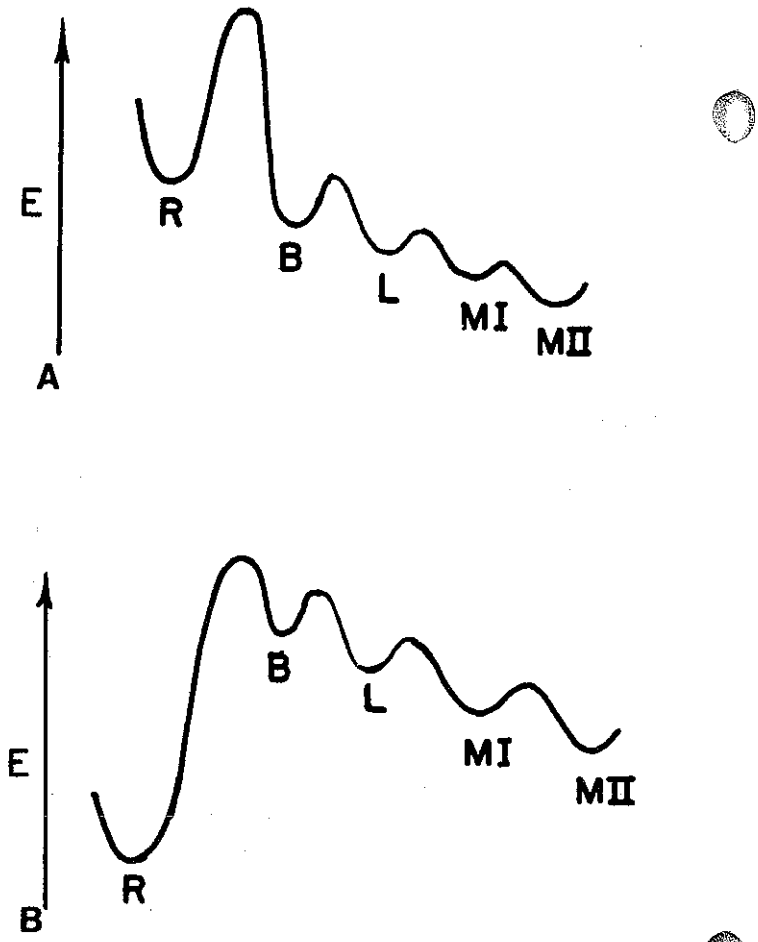

Fig 5 Diagrams depicting two possible schemes for the relative energies of rhodopsin (R) and the intermediates (batho, B; lumi, L; meta I, MI; and meta II, MII) generated as a function of time after light is absorbed. It can be shown that the lower diagram is correct. Here the part of the energy of the photon absorbed by rhodopsin in the first step is stored in B (the batho intermediate). This energizes the subsequent steps in the process.

Unexplained data in an early paper by Wald support this latter hypothesis. In the work he reported on the anomalous behavior of iodopsin (a chicken rhodopsin). In all other rhodopsins studied the six-step reaction depicted in Fig 4 proceeds unidirectionally as shown. The reaction can be slowed — even halted — if the temperature is reduced enough, but at physiological temperatures the reaction is ordinarily a one-way street proceeding inexorably towards the *trans* form. With iodopsin, though, Wald observed a strange effect. When its bathorhodopsin was formed photolytically at 77°K (where it is stable indefinitely) and then heated, it reverted to the *cis*-rhodopsin with a detectable yield. The energy diagram in Fig 5B can explain this anomaly in that the batho form is at a higher energy than the *cis*-retinal [7]. The fact that no other rhodopsin has been found to exhibit this behavior is explainable, too. The energy "well" is too deep, and the thermodynamic barrier is too great, for the reaction to be reversed.

This explanation has been further supported by the spectral properties of the recently discovered bacterial rhodopsin which behaves similarly [7]. Independent of our work, and outside the scope of this article, Honig [18] has concluded from an elegant kinetic argument that all bathorhodopsins are at higher energies than the *cis* forms.

Experimental

Although Raman spectroscopy did not contribute materially to this theory regarding the energetics of the photochemical reaction, it can reveal the mechanism by which the energy is stored and subsequently converted. One type of resonance Raman experiment consists of illuminating a rhodopsin at a low temperature with the same laser as in the photolysis [19]; alternatively, a second laser with an absorption frequency matching the particular rhodopsin intermediate of interest may excite the resonance Raman spectrum [15]. In either case, and depending on the temperature and the exciting wavelength, a photostationary mixture of rhodopsin and its thermal intermediates is produced. Varying the temperature and wavelength changes the relative concentration of the intermediates, and spectral bands can be assigned to the various intermediates as in Fig 4.

Instrumentation for the research is fairly conventional: a Spex Model 1401 double monochromator which is computer-controlled through circuitry designed at Cornell [20]. The source is a Coherent Radiation CR-18 argon ion laser which pumps a tunable dye laser of the same manufacturer; the detector is a cooled RCA C31034 which has a low dark count and an extended red response.

The resonance Raman spectrum of the retinylidene chromophore consists of several bands which are associated with the ~ 17 atoms either directly or indirectly connected to the conjugated portion of the molecule. Where most of these bands originate has not yet been determined but, fortunately, two of the vibrational modes have been unequivocally identified — they correspond to the C=C stretching frequency [21, 22, 23, 24] and the $\rangle C{=}\overset{H}{\overset{|}{\underset{+}{N}}}-$ stretching vibration of the terminal carbon in the polyene containing the nitrogen end group [17, 19]. The terminal carbon-nitrogen vibration, as already mentioned, monitors the state of protonation of the Schiff base linkage, while the C=C stretching is significant because it serves as a monitor of the electron density in the double-bonded portion of the chromophore.

The C=C Stretch

Essentially, the Raman data for rhodopsin is consistent with the interpretation that the greater the electron density in the C=C bond the stronger the bond and the higher its vibrational frequency. With this as a basis the electron density can be plotted as a function of time after a photon interacts with the rhodopsin. Fig 6A and Fig 6B are such plots for bacterial and bovine rhodopsin. As may be seen for both molecules, the C=C stretching frequency is lower after interaction with light because of the delocalization of the electron density. In the batho intermediate this electron-delocalized state stores part of the photon energy to energize the later steps of the process when relocalization occurs [7]. After comparing these data with the weak fluorescence spectra of the retinylidene chromophore observed in our laboratory (with our tunable-laser Raman spectrometer which is also an exceptional fluorometer) [25] we have been able to suggest that the batho intermediate is similar in nature to the excited state of rhodopsin from which it evolves.

Kinetic Resonance Raman Spectra of the C=C Stretch and C=N Stretch

We can now study the protonation of the Schiff base linkage as a function of the electron delocalization and relocalization. This can be accomplished either under photostationary conditions, as already discussed, or with the recently described kinetic resonance Raman spectroscopy (KRRS) [26]. In Fig 7 KRRS follows simultaneously the electron delocalization-relocalization and the protonation of the Schiff base. The resonance raman spectrum of bacterial rhodopsin in Fig 7B is compared to a steady-state spectrum (Fig 7A) obtained under conditions where the predominant species are bacterial rhodopsin (bR_{570}) and an intermediate corresponding to metarhodopsin II (M_{412}). We had previously shown by deuterating the Schiff base that the band at 1646 cm^{-1} was due to the protonated Schiff base linkage and that this band, together with the C=C stretch at 1530 cm^{-1}, arose from bacterial rhodopsin [19]. In addition, our investigations had shown that the band at 1622 cm^{-1} (the carbon-nitrogen vibration for an unprotonated Schiff base) and the C=C stretch at 1567 cm^{-1} corresponded to the M_{412} intermediate. The KRRS in Fig 7B - 7E were obtained with a laser frequency which maximized the resonance enhancement of the M_{412} intermediate. As is clearly seen in these spectra the deprotonation of the Schiff base occurs several milliseconds before the onset of the C=C stretch of the M_{412} intermediate. This indicates that the deprotonation of the Schiff base is probably a direct consequence of the relocalization process (producing the lumi intermediate in bacterial rhodopsin) which follows the formation of the electron-delocalized high-energy batho form. So the deprotonation of the Schiff base appears to be a result of the primary photochemistry, and the ejection of this proton stimulates the conformational changes in the protein

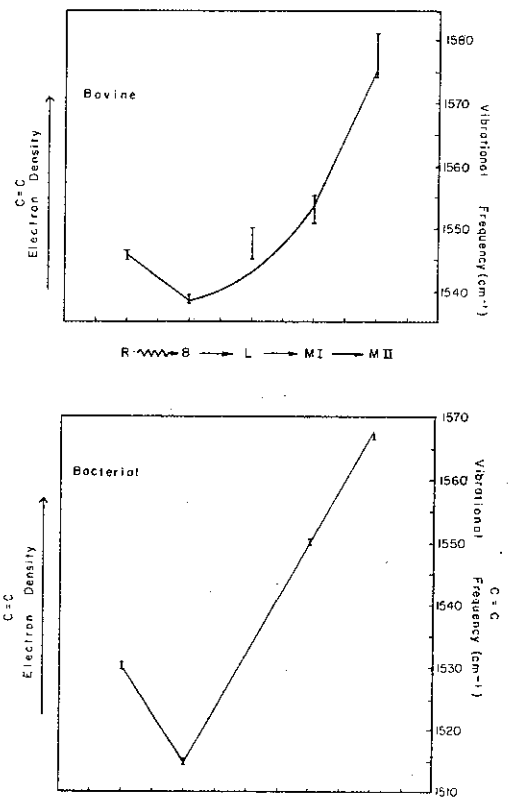

Fig 6 Changes in the electron density of the C=C bond as a function of time after a photon is absorbed by rhodopsin.

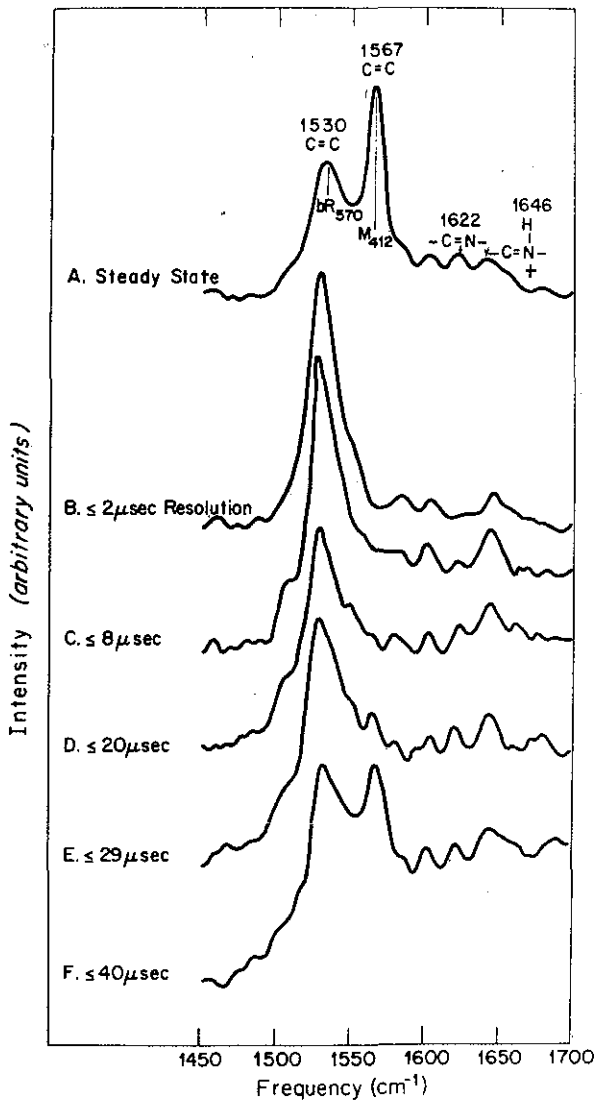

Fig 7 Steady state and kinetic resonance Raman spectra of bacterial rhodopsin over a limited frequency region. These spectra illustrate the dynamics of the deprotonation of the Schiff base linkage as a function of delocalization and relocalization of electron density in the C=C bond.

which yield the metarhodopsin II intermediate. Similar conclusions about the protonation of the Schiff base linkage have been obtained on photoreceptor rhodopsins where the deprotonation of the Schiff base in bovine rhodopsin seems to occur on the same time scale as the generation of a neural response between metarhodopsin I and II.

Thus, as is seen in Fig 8, the interaction of a photon with rhodopsin can be described as an electron delocalization which stores part of the photon's energy to energize the later relocalization of electron density which leads to the ejection of the Schiff base proton [7]. Furthermore, in all photoreceptor rhodopsins the stored photon energy also energizes the formal *cis-trans* isomerization of the retinylidene chromophore. As we have seen, bacterial rhodopsin has identical spectral properties to all other rhodopsins. This suggests that bacterial rhodopsin can accomplish the electron delocalization and relocalization and the proton ejection with one fewer intermediate (see Fig 6), without a detectable isomerization (the chromophore is initially *trans*), and without detaching the chromophore from opsin. In addition this apparently allows bacterial rhodopsin, unlike all other rhodopsins, to regenerate itself thermally in the dark in several milliseconds at physiological temperatures. At first this was puzzling; it suggested that bacterial rhodopsin (presumably a primitive form of rhodopsin) could accomplish more efficiently all the principal spectral transitions seen in photoreceptor rhodopsins. If, however, these spectral similarities are analyzed in the context of biological differences between the rhodopsins, then a very fundamental understanding of the nature of the II-*cis* to *trans* isomerization in vision results.

The II-*cis* to *trans* Isomerization

To appreciate the role of the *cis-trans* isomerization in vision let us consider the biological role of bacterial rhodopsin. Walther Stoeckenius, who discovered bacterial rhodopsin in 1971 [27], has shown that this rhodopsin-like protein is an energy converter for the bacterium [28]. When the bacteria are grown under anaerobic conditions they inhibit the normal mechanisms for energy transduction (which require oxygen) and generate a rhodopsin-like membrane protein which traverses the bacterial cell membrane. This bacterial rhodopsin absorbs light energy for the bacterium, ejects the Schiff base proton, and forms a proton gradient across the bacterial cell membrane [19]. The proton gradient participates in the formation of ATP in the bacterium [29]. Thus bacterial rhodopsin is a light-driven proton pump. Its biological function is to convert light energy into chemical energy. On the other hand, photoreceptor rhodopsins are not energy converters but quantum detectors, and photoreceptors are exceptional quantum detectors, capable of detecting a single photon. For a good quantum detector there must be irreversibility, and this we believe to be the role of the II-*cis* to *trans* isomerization in all photoreceptor rhodopsins [7]. The bacterial rhodopsin, however, has to optimize energy conversion, so the bacteria have evolved a rhodopsin-like protein which minimizes endoenergetic conformational changes and maximizes reversibility [7]. In so doing it sacrifices the high quantum efficiency ($\sim$ 65%) of photoreceptor rhodopsins by approximately a factor of two.

Future Research

Future research will have to focus on the role of the ejected proton in the cellular energy transduction which produces a neural response. In bacterial rhodopsin it is clear that the Schiff base proton plays an important part in stimulating the formation of a proton gradient. It is tempting to suggest, based on the spectral similarities, that rhodopsin in photoreceptor cells also acts as a light-driven proton pump. Unfortunately no evidence exists that supports this hypothesis, and it has already been pointed out that bacterial and photoreceptor rhodopsins have widely differing biological functions. Therefore it is quite possible that bacterial rhodopsin is only a good model system for the photochemistry which is probably similar in all rhodopsins. Contrarily, the proton ejection by the photoreceptor cells may be only the first step in a complicated sequence of reactions which ultimately results in calcium ions' mediation of sodium transport across the plasma membrane. It seems probable that an important element in this energy transduction (amplification) is ATP. In other words, the decrease in ATP after the absorption of a photon [2] may (in some as yet undetermined way) be the bridge between the ejected proton in the disc membrane and the generation (by calcium ions) of the hyperpolarization across the plasma membrane which eventually leads to a neural response.

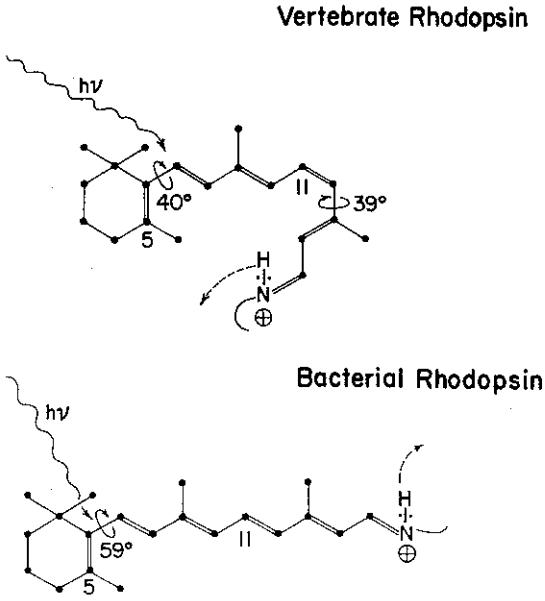

Fig 8 In both vertebrate and bacterial rhodopsin absorption of a photon eventually causes a deprotonation of the Schiff base linkage. Vertebrate rhodopsin, however, is initially in a 11-*cis* (bent) conformation and converts to a *trans* (linear) conformation before the loss of a proton. Bacterial rhodopsin, on the other hand, is *trans* initially and is also *trans* when the deprotonation occurs. It is suggested that the 11-*cis* to *trans* isomerization is essential in all photoreceptor rhodopsins to prevent reversibility of the reaction which is fundamental to the operation of a good quantum detector. Such irreversibility would be a detriment to bacterial rhodopsin which is an energy converter and must maximize reversibility.

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A RAMALOG TACKLES POLLUTION

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A novel application of laser-Raman spectroscopy to a common problem with stack gas control may surprise anyone who thought Raman was a tool better suited to ivory-tower research than to everyday employment in industrial plants. The procedure was developed at Spex in cooperation with a chemical manufacturer who was removing sulfur dioxide gas (SO_2) from smokestack effluents and needed to regenerate the scrubber solution efficiently.

The scrubber system depends on an aqueous phosphate buffer which absorbs the sulfur dioxide, converting it to bisulfite ions (HSO_3^-) in solution. After the bisulfite builds up, the solution can be recycled by treating it with hydrogen sulfide (H_2S) to precipitate sulfur. The method is a good one, but it is vulnerable to inefficient regeneration of the buffer solution; too little hydrogen sulfide and some bisulfite will remain; too much hydrogen sulfide and the excess will escape into the atmosphere as pollution.

To complicate the situation, the complexity of the mixture means that techniques developed for simple solutions won't work. Redox titrations [1] are not satisfactory because interfering reducing agents (such as polythionates and thiosulfate) are present and because air oxidizes neutral and alkaline solutions; specific ion electrodes [2] are handicapped by the high ionic strength as well as by interferences; the spectrophotometric procedure [3] will work for bisulfite, but it takes so long as to be impractical for continuous processing, and other sulfur-containing anions must be determined separately.

Since Raman exploits differences in the vibrational spectra of various anions in aqueous solution, it was deemed a likely candidate for this application.

TYPICALLY, the solution to be analyzed is approximately 0.5 M each in KH_2PO_4 and K_2HPO_4 and contains 50 - 70 g/l sulfate as Na_2SO_4, 20 g/l thiosulfate as $Na_2S_2O_3$, 1 - 8 g/l SO_2 as $NaHSO_3$, and various polythionates in varying concentrations. Raman spectra of solutions of Na_2SO_4, Na_2SO_3, and Na_3PO_4 (pH 9) exhibit such overlap of the ν_1 bands in the 900 - 1050 cm^{-1} region that quantitative analysis is impossible. Bands in other regions of the spectra are too weak to be of value.

At lower pH (approximately 4.0) the ν_1 bands of sulfate and phosphate are resolved at 890 and 980 cm^{-1}, presumably due to the protonation of phosphate to other species (primarily $H_2PO_4^-$). The bands of bisulfite cannot be distinguished from those of the sulfate and phosphate ions which are present at much higher concentrations.

Acidifying a solution of sodium bisulfite to a pH around 1.5, however, produces free sulfur dioxide in solution according to Eq 1, and an interference-free band is obtained.

$$HSO_3^- + H^+ \rightleftharpoons H_2SO_3 \rightleftharpoons H_2O + SO_2 \qquad (1)$$

Sulfur dioxide is a bent molecule with S=O double bonds. It has two stretching vibrations, ν_1 at about 1157 cm^{-1} and ν_3 at about 1341 cm^{-1}; the symmetrical mode ν_1 is Raman active [4]. The 1157 cm^{-1} band is sufficiently removed from the strong sulfate and phosphate bands to allow a quantitative determination, as the only signal in this region is attributed to Raman-inactive bands of sulfate and phosphate (ν_3) and constitutes no interference. (See figure.)

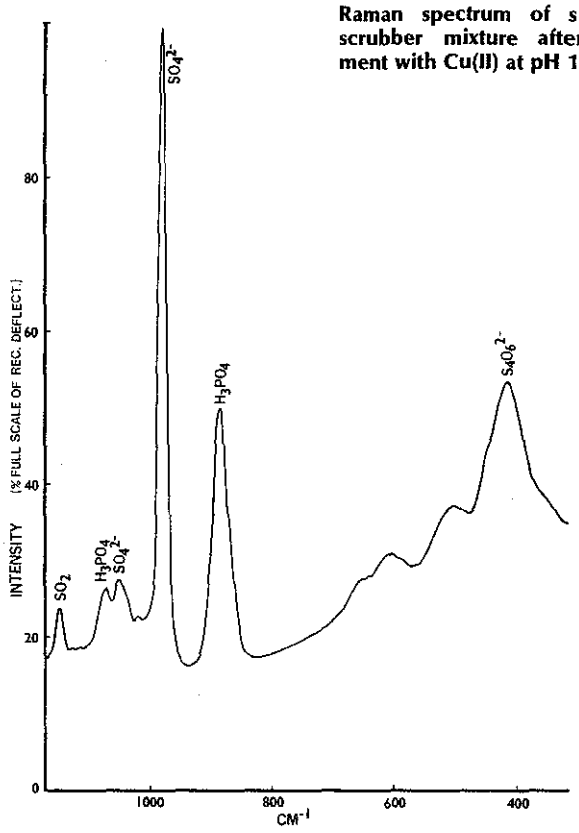
The major obstacle to the method is the high concentration of thiosulfate because the ion reacts instantaneously with acid as shown in Eq 2.

$$S_2O_3^{2-} + H^+ \longrightarrow S + SO_2 + OH^- \qquad (2)$$

It is therefore essential to complex or convert the thiosulfate to an inert, or noninterfering, species without destroying or creating any bisulfite. A search of redox reactions revealed that Cu(II) reacts with thiosulfate to convert it to *tetra*thionate according to Eq 3.

$$6S_2O_3^{2-} + 2Cu^{2+} \longrightarrow 2Cu(S_2O_3)_2^{3-} + S_4O_6^{2-} \qquad (3)$$

It is necessary to acidify the mixture to pH 4 before adding Cu(II); otherwise $CuHPO_4$ or $Cu(H_2PO_4)_2$ will precipitate. Since oxidation of the thiosulfate yields a colorless Cu(I) ion



Raman spectrum of synthetic scrubber mixture after treatment with Cu(II) at pH 1.5

the complete removal of free thiosulfate is evidenced by a change from colorless to pale green after sufficient Cu(II) is added. The solution is then acidified without decomposition.

THE method was tested on synthetic mixtures with compositions typical of scrubber solutions; it proved entirely satisfactory. The calibration curves run were for sulfur dioxide (1 - 8 g/l) and sulfate (45 - 70 g/l) and were linear. Other species could be determined just as easily.

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