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**Visual pigments and bacteriorhodopsin: A resonance Raman
study**

Rath, Parshuram, Ph.D.

City University of New York, 1989

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**VISUAL PIGMENTS AND BACTERIORHODOPSIN:
A RESONANCE RAMAN STUDY**

by

PARSHURAM RATH

A dissertation submitted to the Graduate Faculty
in Physics in partial fulfillment of the requirements
for the degree of Doctor of Philosophy,
The City University of New York.

1989

The manuscript has been read and accepted for the Graduate Faculty in Physics in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

VISUAL PIGMENTS AND BACTERIORHODOPSIN: A RESONANCE RAMAN STUDY

by

PARSHURAM RATH

Adviser: Professor Robert H. Callender

In an effort to achieve better understanding of the mechanism of visual transduction in invertebrates, we have studied two visual pigments: the ultraviolet absorbing invertebrate visual pigment of owlfly (*Ascalaphus macaronius*) and the visible absorbing pigment of octopus (*Paroctopus deflini*). Studies on the owlfly pigment suggest that a protonated chromophore is essential for vision to occur and that both the vertebrates and invertebrates share a common mechanism of visual transduction. We have worked towards resolving the observed differences in the low frequency HOOP region of octopus and bovine bathorhodopsins. Resonance Raman studies of C₁₀-D and C₁₂-D retinal regenerated octopus visual pigments suggest that the location of a negatively charged protein residue near C₁₂ of octopus bathorhodopsin chromophore is unlikely.

We have also studied another light transducing retinal pigment, bacteriorhodopsin. We have regenerated bacteriorhodopsin from opsin and all-*trans* retinal and an intermediate of regeneration was studied. Results suggest that the shift in color of the retinal from 380 nm to 570 nm (in the reconstituted pigment) is achieved through several steps. An analogue of bacteriorhodopsin, 13-bromo-bR, was also studied. We have looked into the various intermediates of its photocycle. Our data suggest that although the thermal isomerization of this chromophore is hindered, its photoisomerization is quite efficient. We have also looked into the possible existence of different pH dependent forms of bacteriorhodopsin. Our Raman data are in disagreement with such hypothesis.

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Foreword

The early work in vertebrate vision was pioneered by Wald at the Harvard University in 1950s and the studies were made primarily on bovine visual pigment rhodopsin. Since then many other visual pigments have been studied to a certain extent, both in vertebrates and invertebrates. To date, even after more than three decades of intensive research, the exact mechanism of visual transduction is not completely understood. Application of lasers in this field and the studies of another light transducing retinal-pigment system, bacteriorhodopsin, has helped further our understanding of the mechanism of vision. Our goal in this thesis was to extend our knowledge in this direction and investigate on a common model for the process of vision across various species, both vertebrates and invertebrates. We have studied the UV absorbing visual pigment of an invertebrate and worked towards resolving some of the differences observed in the Raman spectra of octopus and bovine visual pigments. We have also studied to some extent the spectroscopic differences of bacteriorhodopsin at different pH conditions. An intermediate of the bacteriorhodopsin regeneration is studied and we looked into the photocyclic capability of a bacteriorhodopsin analogue, the 13-bromo-bR.

Chapter 1 introduces to the basic concept behind Raman and resonance Raman spectroscopy. Introduction to the biological systems studied in this work, rhodopsin and bacteriorhodopsin, are also given in this chapter. The next chapter describes various experimental setups and the procedures of data collection, their storage and manipulation. Chapter 3 is devoted to the study of an intermediate in bacteriorhodopsin regeneration. Chapter 4 details the resonance Raman study of the UV

sensitive invertebrate visual pigment of *Ascalaphus macaronius*. Studies on the deuterium labelled octopus visual pigment is given in chapter 5. Chapter 6 describes the studies made on bacteriorhodopsin at alkaline pH and the final chapter discusses about an analogue of bacteriorhodopsin, the 13-bromo-bR.

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LIST OF SYMBOLS AND ACRONYMS

bR	Bacteriorhodopsin
bR ^{DA}	Dark-adapted bacteriorhodopsin
bR ^{LA}	Light-adapted bacteriorhodopsin
bR ₄₅₀	The 450-nm absorbing intermediate of bR regeneration
bR ₄₆₀	bR species above pH 11.5
bR ₄₈₀	bR in DMSO/delipidated bR
FWHM	Full Width at Half Maximum
K ₆₁₀	The K photo-intermediate of native bR
$\lambda_{\max}$	Wavelength at the maximum absorption
L ₅₅₀	The L photo-intermediate of native bR
M ₄₁₂	The M photo-intermediate of native bR
mM	millimolar
min	minute
mol	mole
μ l	microliter
μ or μ m	micrometer (micron)
nm	nanometer
O ₆₄₀	The O photo-intermediate of native bR
OD	Optical Density unit
ps	picosecond
PSB	Protonated Schiff base of retinal
rpm	revolutions per minute
SB	Schiff base of retinal
sr	Steradian (solid angle unit)
UV	Ultra Violet

CHAPTER 1

Introduction

1.1 Raman Effect

The Raman effect is an inelastic light scattering process in which a change in the frequency of light is observed. When a molecule is irradiated with light (photons) of frequency ν_L (energy $h\nu_L$), a portion of the incident light is scattered. The scattered light intensity is directly proportional to the fourth power of ν_L . In the scattering process, the energy of the majority of the photons remains unchanged. This elastic process where the photon energy is conserved is described as *Raleigh scattering*. Some of the incident photons exchange energy with the molecule and appear either on the higher energy or lower energy side of the Raleigh scattered photons. These frequency shifted photons are termed Raman photons. The scattering process giving rise to photons with energy lower than the incident photon energy is known as *Stokes Raman scattering*. *Anti-Stokes Raman scattering* process gives rise to photons with energy higher than that of incident photons.

1.1 a. A classical description

The scattering process can be qualitatively understood in a classical description. Light is considered as oscillating electric and magnetic fields. When a molecule is irradiated, the electric vector of light disturbs the electron distribution and induces a dipole in the molecule. The dipole moment is proportional to the electric field and is given by the relation

$$\mathbf{p}(t) = \alpha(t)\mathbf{E}(t) \quad (1)$$

where $\mathbf{p}(t)$ is the dipole moment, $\alpha(t)$ is the electric polarizability of the molecule and is a tensor and $\mathbf{E}(t)$ is the electric field. If the light incident on the molecule is of frequency ν_L and its electric vector points in z direction, the oscillating electric field $\mathbf{E}(t)$ can be written as

$$\mathbf{E}(t) = E_z \cos(2\pi\nu_L t) \quad (2)$$

where E_z is the amplitude of oscillation.

Thus the z component of the dipole moment $\mathbf{p}(t)$ is given by

$$p_z(t) = \alpha_{zz}(t) E_z \cos(2\pi\nu_L t) \quad (3)$$

Vibration of the molecule causes oscillation of α . In the simple case of a diatomic molecule, the polarizability depends linearly on the nuclear coordinates $q(t)$ and can be expanded around the equilibrium configuration of the molecule as

$$\alpha_{zz}(t) = \alpha_{zz}(0) + \alpha'_{zz} \Delta q(t) \quad (4)$$

where $\alpha_{zz}(0)$ is the equilibrium value of polarizability and α'_{zz} is the first order derivative of α_{zz} with respect to the nuclear coordinates q . The time dependence of $q(t)$ due to molecular vibration is given by the relation

$$q(t) = q_0 \cos(2\pi\nu_v t) \quad (5)$$

where ν_v is the molecular vibrational frequency, q_0 is the amplitude of $q(t)$.

Now the time dependence of the dipole moment can be explicitly written as

$$\begin{aligned} p_z(t) &= \alpha_{zz}(t) E_z \cos(2\pi\nu_L t) = (\alpha_{zz}(0) + \alpha'_{zz} \Delta q(t)) E_z \cos(2\pi\nu_L t) \\ &= \alpha_{zz}(0) E_z \cos(2\pi\nu_L t) + (1/2)\alpha'_{zz} \Delta q_0 E_z \cos 2\pi(\nu_L + \nu_v)t \\ &\quad + (1/2)\alpha'_{zz} \Delta q_0 E_z \cos 2\pi(\nu_L - \nu_v)t \end{aligned} \quad (6)$$

In classical physics, an oscillating dipole radiates energy and the total power radiated (P) is given by (Jackson, 1975)

$$P(t) = (8/3)(\pi^4/c^3)(\nu_L)^4 |p(t)|^2 \quad (7)$$

When equation (6) is incorporated into (7), we obtain the total power radiated from an oscillating dipole in terms of the incident light frequency and the vibrational frequency of the molecule. From the above equation (7) it can be seen that the intensity of the scattered light varies as the fourth power of the incident light frequency.

As a result of the first term in (6), light of incident frequency ν_L will be emitted and observed in all directions. This is the phenomenon of *Raleigh scattering*. The scattered light arising from the second term is known as *anti-Stokes Raman scattering*. The third term will give light quanta of frequency $\nu_L - \nu_v$, and is known as *Stokes Raman scattering*. The coefficient of the cosine factor in each of these terms give the intensity of the Raleigh or Raman scattering.

When the molecule is excited with a light of frequency close to its absorption maximum, a large increase in the intensity of the Raman lines is observed. This resonance phenomenon, known as the *resonance Raman effect*, cannot be explained through the classical approach outlined above. A semi-classical approach to the problem, discussed below, explains the observed resonance effect.

1.1b. Semi-classical approach and resonance Raman effect

After the discovery of Raman effect (Raman and Krishnan, 1928), the basic theory of Raman scattering was developed by Placzek (Placzek, 1934). This theory was first applied in detail to molecular systems by Albrecht (Albrecht, 1961) and since then a considerable amount of theoretical work has been done in the field of Raman and resonance Raman scattering (Tang and Albrecht, 1968, 1970; Peticolas *et al.*, 1970; Mingardi and Siebrand, 1973, 1974, 1975; Johnson and Peticolas, 1976). In the semi-classical Placzek's theory of Raman scattering, the radiation field is described classically and the molecule is treated quantum mechanically (see figure 1.1). The Raman intensity $I_{g^0 \rightarrow g^1}$, for the transition from the lowest vibrational state $i=0$ to the vibrational state $i=1$ [Stokes process, figure 1.1(a)], both in the ground electronic state g , is proportional to the square of the corresponding term in the polarizability tensor and is given by (just as in the case of classical description given above)

$$I_{g^0 \rightarrow g^1} = \frac{2^7 \pi^5}{9 c^4} I_0 (\nu_s)^4 \sum_{\rho\sigma} \left| \alpha_{g^0, g^1}^{\rho\sigma}(\nu_L) \right|^2 \quad (8)$$

where I_0 is the incident flux, ν_L is the incident light (laser) frequency, $\nu_s = \nu_L - \nu_0$ is the scattered light frequency (see figure 1.1 for all the notations used), where ν_0 is the energy separation between the vibrational levels g_0 and g_1 .

The $\alpha_{g_0, g_1}^{\rho\sigma}(\nu_L)$ component of the polarizability tensor is given by

$$\alpha_{g_0, g_1}^{\rho\sigma}(\nu_L) = \sum_{ej} \left[\frac{\langle g_0 | M_{eg}^{\rho}(Q) | ej \rangle \langle ej | M_{eg}^{\sigma}(Q) | g_1 \rangle}{\Delta E_{eg} - h\nu_L + \epsilon_j + i\Gamma_{ej}} + \frac{\langle g_0 | M_{eg}^{\sigma}(Q) | ej \rangle \langle ej | M_{eg}^{\rho}(Q) | g_1 \rangle}{\Delta E_{eg} + h\nu_s + \epsilon_j + i\Gamma_{ej}} \right] \quad (9)$$

where $|ej\rangle$ denotes the j^{th} vibrational level in the e^{th} excited state (figure 1.1), ϵ_j is the vibrational energy of that level, and ΔE_{eg} is the energy difference between the ground and excited electronic state. $M_{eg}(Q)$ is the electronic transition dipole moment expressed as a function of the nuclear coordinates Q , ρ and σ are the polarization vectors of the incident and scattered light. The factor $i\Gamma_{ej}$ in the denominators of equation (9) is the phenomenological line width factor which takes in to account the finite lifetime of each of the molecular state.

It can be seen that when $\Delta E_{eg} \approx h\nu_L$, the denominator in the first term in (9) becomes small giving rise to a large enhancement of Raman intensity (resonance Raman effect). In this case the sum over electronic state may reduce to a single term in which case the vibrational sum over j pertains to the vibronic states of the excited electronic state in resonance with the incident photon. The second term in (9) makes, in general, negligible contribution to the Raman intensity and can be neglected in the resonance

condition. In the first approximation, the electronic transition dipoles are assumed independent of nuclear coordinates, i.e. $M_{eg}(Q) = M_{eg}(0)$, and we obtain

$$\alpha_{g0, g1}^{\rho\sigma}(\nu_L) = M_{eg}^{\rho}(0) M_{eg}^{\sigma}(0) \sum_j \frac{\langle g0|ej\rangle\langle ej|g1\rangle}{\Delta E_{eg} - h\nu_L + \epsilon_j + i\Gamma_{ej}} \quad (10)$$

This is the so called Albrecht A term discussed first by Albrecht (1961). In this term (10), $\langle g0|ej\rangle$ and $\langle ej|g1\rangle$ are overlap integrals between vibrational wave functions of the ground and excited states. The factors $\langle g0|ej\rangle$ are the Franck-Condon integrals which determine the appearance of vibrational progressions in absorption or fluorescence spectroscopy. Raman intensity goes to zero when these terms are small. This means that the A term will have significant contribution to the Raman intensity only for the vibrations that appear as progressions in electronic spectra.

In the off-resonance situation, particularly when $\Delta E_{eg} \gg h\nu_L$, the polarizability is independent of the incident light frequency but involves contributions from all excited vibronic and electronic states. The A term, in this case, goes to zero and an expansion of $M_{eg}(Q)$ about $Q = Q_{eq}$, Herzberg-Teller approximation, leads to a so called B term. The B term is the major contributor to the Raman intensity when the laser photon is far from resonance with the excited state (Tang and Albrecht, 1970; Albrecht, 1961; Inagaki *et al.*, 1974; Johnson and Peticolas, 1976). The contribution of the B term to the resonance cross section may not be ignored in all situations but the A term is probably the major contributor to the resonance Raman cross section of visual pigments.

1.1c. Application to biochemical studies

Thus we have seen that Raman spectra are rich in information about the vibrational motions (normal modes) of the molecule, and because these vibrations are sensitive to chemical changes, the vibrational spectrum can be used as a monitor of molecular chemistry. The vibrational energy separations are in the range of infrared frequency, thus infrared absorption spectra of molecules are similar to the Raman spectra. In some molecules, some of the infrared active vibrational modes are not seen in their Raman spectra and vice versa. In such situations, Raman spectra are supplemented with the infrared spectra for a complete vibrational analysis of the molecule.

The band intensities in resonance Raman spectra are much larger than those in non resonant spectra, some 8 orders of magnitude greater for visual pigments (Callender and Honig, 1977). Apart from making spectra intrinsically easier to detect, the resonance Raman effect introduces the possibility of selectively probing the vibrational spectrum of a small but key part of an extremely complex biological system, e.g. visual pigments. This is achieved by choosing a system that contains a chromophore of interest which absorbs photons in near visible region. By exciting the system with light that can be selectively absorbed by the chromophore the resonance Raman spectrum of that chromophore may thereby be obtained uncluttered by the normal Raman spectrum from the rest of the system, which, being orders of magnitude less intense, is lost in the spectral background.

1.1 d. Merits and liabilities of this technique for biochemical studies

There are many advantages and disadvantages associated with the use of Raman/resonance Raman spectroscopy for the studies of biological and biochemical systems. Some of them are outlined below.

Advantages

1. Since water is a very weak Raman scatterer, Raman spectra may be obtained from molecules in aqueous solutions. This is very advantageous for the studies of biological systems.
2. Very small sample volumes are needed for Raman studies. The 'active volume' of Raman scattering is governed by the size of the focussed laser beam, typically $0.005 \times 0.2 \text{ cm}^2$, and the sample need only fill this volume.
3. Resonance Raman spectra can be obtained from micromolar concentrations of the sample, just like any other biochemical studies and the sample is reusable after Raman studies.
4. Raman spectra can be recorded from samples under diverse experimental conditions. Raman studies can be made with samples at any temperature, pressure or other physical conditions as long as, of course, the experimental condition does not cause sample degradation.
5. Liquids, solids, solutions, gases, films, fibers and single crystals are amenable to examination.
6. Unlike infrared studies, Raman studies need almost no sample preparation.
7. It affords a non-contact (non chemical) sample analysis. No chemical changes in the sample has to be made for its identification.

8. Complete Raman spectrum of a sample can be obtained in just one second when coupled with diode array or nondispersive techniques.
9. Since the time scale of Raman scattering is in the order of 10^{-11} sec it can be used for kinetic studies of most biochemical reactions.
10. Resonance Raman can be used for selective study of a complex biological system.

Disadvantages

1. Raman and even resonance Raman effect are highly improbable physical processes, and the Raman spectrum is easily obscured by sample fluorescence.
2. The high photon flux of the laser beam can produce unwanted photochemical effects.
3. Normal Raman spectroscopy requires sample concentrations which are considered rather high by biochemists.
4. Sample solution should be highly homogeneous for normal Raman studies.

1.2. Vision and Visual pigments

Vision is associated with retinal cells transducing incident photon energy into a neural signal. Vertebrates with color vision have retinal cells that are either rod shaped (rods) or cone shaped (cones). Cones are responsible for color vision and rods are extremely efficient light detecting cells responsible for vision in dark. Typically there are 10^8 rods in a vertebrate retina. Each rod cell is about $6\mu\text{m}$ in diameter and $\sim 60\mu\text{m}$ long. A sketch of the vertebrate rod is shown in figure 1.2. The outer segment of the rod cell is filled with about 2000 sacs known as discs. Rhodopsin

molecules which are the light absorbing components of the rods are embedded in a specialized membrane in these discs. The electrical response of rods saturates at the light level which corresponds to about 7000 single-photons absorption/sec/rod. This light level closely matches the lowest intensities at which cone cells can be excited. The visual transduction process is carried out by rods in dim light and cones take over in bright light (when the response of rods saturates).

1.2a. Bovine visual pigment and vision in vertebrates

The most studied vertebrate visual system is bovine rhodopsin. The membranes in the discs of the vertebrate's rod outer segment consists of about 40% (dry weight) lipids and 60% proteins. Most of these proteins are rhodopsins (Papermaster and Dryer, 1974). Removal of the lipid components affects some of the properties of rhodopsin, namely its thermal stability (Daemen, 1973) and regeneration (Zorn and Futterman, 1971; Hong and Hubbell, 1973). The density of rhodopsin molecules in the disc membrane is quite high (see e.g. Bridges, 1970). Rhodopsin is a glycoprotein consisting of a 11-*cis* retinal chromophore (figure 1.3) covalently bound to a protein (opsin) (Hubbard and Kropf, 1958). The chromophore-opsin linkage in rhodopsin is a protonated Schiff base bond (Lewis *et al.*, 1973; Oseroff and Callender, 1974). The stability of the Schiff base linkage over a wide range of solution pH (Radding and Wald, 1956) and its resistance to sodium borohydride (Bownds and Wald, 1965) suggests that the Schiff base linkage is enfolded inside a three-dimensional frame of the polypeptide chain.

The amino acid sequence of bovine rhodopsin is known (Ovchinnikov, 1982; Hargrave *et al.*, 1983). Bovine rhodopsin is a 36000

dalton protein containing 348 amino acids. The binding site of the chromophore is the ϵ -amino group of Lys₂₉₆ (Fager *et al.*, 1972; DeGrip *et al.*, 1973; also see Ovchinnikov, 1987).

Bovine rhodopsin has a broad absorption band centered at 498 nm ($\lambda_{\text{max}} = 498$ nm). Absorption of a photon isomerizes the chromophore of rhodopsin (Yoshizawa and Wald, 1963) leading to the detachment of chromophore from the opsin. This process is called *bleaching* since rhodopsin absorbs in visible while the final products, retinal and opsin, absorb in UV. Details of the bleaching sequence (figure 1.4) have been elucidated by Wald (1968). Photochemical kinetics studies using ultra-short laser pulses have shown that under physiological conditions bathorhodopsin is formed in less than a picosecond of photon absorption by rhodopsin (Busch *et al.*, 1972; Peters *et al.*, 1977; also see Lewis and Del Priore, 1988). In the absence of light, bathorhodopsin is bleached in just a few milliseconds (figure 1.4). Bathorhodopsin can also absorb a photon and convert back to either rhodopsin or to another pigment isorhodopsin (Yoshizawa and Wald, 1963). The isorhodopsin contains the 9-*cis* retinal chromophore and follows the same bleaching sequence as rhodopsin. The intermediates of bleaching can be studied by trapping them at low temperatures.

Vibrational Raman spectroscopy has been used for identifying the isomeric conformations of visual pigment chromophores. Resonance Raman studies have confirmed that the chromophore in rhodopsin is a 11-*cis* isomer of retinal (figure 1.3) linked to the opsin via a protonated Schiff base linkage (Oseroff and Callender, 1974; Callender *et al.*, 1976). Raman spectra of bovine bathorhodopsin ($\lambda_{\text{max}} = 543$ nm) have been obtained (Eyring and Mathies, 1979; Aton *et al.*, 1980; Narva and Callender, 1980).

Bathorhodopsin is also linked to the opsin via a protonated Schiff base linkage and there is a large body of evidence that its chromophore has a distorted all-*trans* configuration (Eyring *et al.*, 1980).

The stages of metarhodopsin I (meta-I) ($\lambda_{\text{max}} = 478 \text{ nm}$) and metarhodopsin II (meta-II) ($\lambda_{\text{max}} = 380 \text{ nm}$) are quite interesting because they have been correlated with neural activity. Meta-I and meta-II are in thermal equilibrium with each other. The equilibrium depends on the temperature, solution pH and solvent but it is not affected by light (Matthews *et al.*, 1963). Increase in either temperature, acidity or ionic strength shifts the equilibrium towards meta-II. Raman studies have shown that the chromophores in both of the metarhodopsins are in all-*trans* (figure 1.3) configuration (Doukas *et al.*, 1978). The Schiff base linkage in meta-I is protonated while in meta-II it is unprotonated.

Metarhodopsin II is believed to play a catalytic role triggering a cascade of chemical reactions leading to the generation of a neural pulse. It has been proposed that meta-II complexes with a GDP binding protein called transducin (T) (Stryer, 1986). Transducin has three subunits, T_{α} , T_{β} , T_{γ} . In the dark, transducin is generally bound to GDP. Meta-II catalyzes the exchange of GDP to GTP and produces the complex meta-II.T.GTP. The next step in the chemical reaction is the dissociation of meta-II.T.GTP into T_{α} .GTP. A single molecule of meta-II can catalyze the release of about 500 T_{α} .GTP molecules. The T_{α} .GTP complex binds with a molecule of PDE (phosphodiesterase, an enzyme) to form another complex T_{α} .GTP.PDE $_{\alpha\beta}$. This latter complex catalyzes the breakup of cGMP. A low level of cGMP results in the closure of sodium ion channels into the rods thus hyperpolarizing the cell. An electrical signal (neural pulse) is generated and vision is completed. This chemical step is associated with a

high amplification factor. One molecule of meta-II can catalyze the breakup of as many as 400,000 cGMP molecules before undergoing a self-timed deactivation.

The energy needed to drive the photochemical reactions in rhodopsin is provided by the incident photons. It has been found that ~32kcal/mol of energy is stored during the rhodopsin to bathorhodopsin transition (Cooper *et al.*, 1979; Schick *et al.*, 1987). This translates to about 67% of the incident photon energy. It is still not clear how this energy is stored in bathorhodopsin. Several models have been proposed in the literatures to explain the mechanism of energy storage in visual pigments. Absorption of a photon is accompanied by the isomerization of the protonated (charged) chromophore in rhodopsin. This leads to a suggestion that the photon energy may be captured in visual pigments through electrostatic interactions between the charged chromophore and the surrounding protein. This suggestion is incorporated in the point-charge model proposed by Honig *et al.* (1979a, 1979b), in which isomerization of the chromophore about C₁₁=C₁₂ double bond flips the C=NH⁺ moiety away from its protein counter-ion (figure 1.5). The estimated energy storage in this model could easily be more than 20kcal/mol. Isomerization leading to the separation of Schiff base charge from its protein counter-ion could also account for the observed red shift in the absorption (498 nm in rhodopsin to 543 nm in bathorhodopsin). In this model, the Schiff base environment of bathorhodopsin is assumed different from that of rhodopsin. This question has recently been examined by Deng and Callender (1987) and by Rodman Gilson *et al.* (1988) whose calculations show that any significant change in hydrogen bonding environment should alter the Schiff base frequency. Since both rhodopsin and bathorhodopsin have the same Schiff base

frequency (1657 cm^{-1}), no change in the Schiff base environment upon isomerization is expected. In the light of these observations several modifications to the above model have been proposed (see e.g. Deng and Callender, 1987). Theoretical calculations (Deng and Callender, 1987; Rodman Gilson *et al.*, 1988; Birge *et al.*, 1988) show that a substantial amount of the photon energy can be stored by the motion of the chromophore in the charged environment of the protein.

1.2b. Octopus visual pigment

The photochemical reactions of the invertebrate rhodopsin of octopus is very much similar to that of the vertebrate bovine rhodopsin (see Table-I). Illumination of octopus rhodopsin ($\lambda_{\text{max}} = 472\text{ nm}$) results in the formation of the primary photoproduct bathorhodopsin ($\lambda_{\text{max}} = 540\text{ nm}$). At physiological temperatures, bathorhodopsin undergoes a series of thermal (dark) reactions leading to the formation of a stable species, acid metarhodopsin ($\lambda_{\text{max}} = 514\text{ nm}$) or alkaline metarhodopsin ($\lambda_{\text{max}} = 376\text{ nm}$), depending on the solution pH. At low temperature (e.g. 80K), however, the thermal reactions are blocked, and continuous illumination results in significant accumulation of the artificial 9-*cis* pigment, isorhodopsin ($\lambda_{\text{max}} = 460\text{ nm}$), in the photostationary mixture besides rhodopsin and bathorhodopsin. The relative amounts of these species in a photostationary mixture depend upon the illuminating wavelength. At liquid helium temperatures ($\sim 10\text{K}$), another species, hypsorhodopsin ($\lambda_{\text{max}} = 440\text{ nm}$), has also been observed in the photostationary mixture of octopus rhodopsin. This species was shown to decay to bathorhodopsin upon raising the temperature to 70K (Tsuda *et al.*, 1980). Calorimetric experiments (Cooper *et al.*, 1986) show that nearly the same amount of

light energy is converted to chemical energy in the bovine and octopus systems. Also, resonance Raman experiments (Pande *et al.*, 1987) and chemical extraction data show that the octopus rhodopsin and bathorhodopsin have 11-*cis* and all-*trans* chromophores, respectively, linked to the opsin via protonated Schiff base linkage, just like their bovine counterparts. Resonance Raman spectroscopy has been used to characterize the acidic and alkaline metarhodopsins of octopus visual pigment (Kitagawa & Tsuda, 1980), and the chromophore configuration was suggested to be essentially all-*trans*. The chromophore in the octopus hypsorhodopsin has been suggested to be in some torsionally distorted state, perhaps being between the 11-*cis* and the all-*trans* isomeric configurations (Pande *et al.*, 1984).

There are some interesting differences in the photochemical behavior of vertebrate and invertebrate pigments. For example, even though octopus rhodopsin appears to undergo a similar sequence of changes immediately following photoexcitation as the bovine rhodopsin does, forming mostly bathorhodopsin, it differs from the latter in that the chromophore-protein complex never dissociates (Tsuda, 1979, 1982). One or the other stable species, acid metarhodopsin or alkaline metarhodopsin, is formed depending on the solution pH. It should be noted that the meta-I and meta-II intermediates of the bovine photopigment are quite similar to the octopus acidic or alkaline metarhodopsin, respectively, in their absorption maxima as well as the nature of the Schiff base linkage, the former being protonated while the latter is unprotonated (see Table-I). The bovine metarhodopsins are, however, unstable and undergo hydrolysis. Furthermore, the octopus acid metarhodopsin can be converted back to rhodopsin at ~10°C by using orange light (Suzuki *et al.*, 1976). Also,

unlike vertebrate rhodopsins, the cephalopod rhodopsins produce large amounts of hypsorhodopsin (Tsuda *et al.*, 1980b), besides the usual rhodopsin and bathorhodopsin, upon illumination at liquid helium temperatures.

1.2c. Visual pigment of *Ascalaphus macaronius*

The spectroscopic nature of visual pigments is very diverse. Their absorptions span from ultraviolet in some bees to near infrared in snakes. Example of an ultraviolet absorbing visual pigment is the rhodopsin of *Ascalaphus macaronius* (owlfly) which absorbs at 345 nm in UV. It was shown some time ago (Paulsen and Schwemer, 1972) that the rhodopsin in the UV-sensitive eyes of owlfly contains an 11-*cis* retinal chromophore.

Upon irradiation, owlfly rhodopsin undergoes a series of dark reactions shown in figure 1.6. Although extensive data on the photochemical intermediates of this system are not yet available, it appears to undergo reactions similar to the bovine (vertebrate) and cephalopod (invertebrate) rhodopsins. Absorption of photon converts rhodopsin to the supposed (yet unobserved) primary photoproduct, bathorhodopsin, which thermally decays to metarhodopsin via lumirhodopsin. Like the other invertebrates, squid and octopus, the owlfly metarhodopsin equilibrates between the acid-base pair, the acid metarhodopsin and the alkaline metarhodopsin. Under physiological conditions, the predominant photoproduct of owlfly rhodopsin is acid metarhodopsin. Unlike vertebrate (bovine) metarhodopsins which are unstable and hydrolyze to retinal and opsin, the metarhodopsin of owlfly is stable and can be photochemically converted to the primary pigment rhodopsin in a quantitative manner using a suitable color of light. Vibrational spectroscopic data of all the photo

(thermal)-intermediates of owlfly visual pigment are not yet available. The mechanism of photo-transduction in UV-absorbing visual pigments is also poorly understood.

1.3. Bacteriorhodopsin

Besides bovine rhodopsin, a very well studied prosthetic pigment is the purple membrane of the microorganism *Halobacterium halobium*, often referred to as bacteriorhodopsin (Oesterhelt and Stoeckenius, 1971) or simply bR. When oxygen supply is insufficient, *H. halobium* uses bR to transduce photon energy to support metabolic activities.

The microorganism *Halobacterium halobium* is a rod shaped prokaryote of about 0.5 μ m in diameter and 5 μ m long. It grows in natural brines where the salt concentration is at or near saturation. Under arid atmospheric condition it develops patches of purple colored membranes known as the 'purple membranes'. Bacteriorhodopsin is the only protein present in this specialized membrane. It is a 26000 dalton retinal protein containing retinal covalently bound to the protein (opsin) via a Schiff base linkage (for a review see e.g. Stoeckenius and Bogomolni, 1982). For *Halobacteria*, bR acts as a light energy-transducing pigment which absorbs light and stores it in the form of an electro-chemical proton gradient across the cell membrane. This energy is later utilized to support various metabolic activities of the cell and also probably for locomotion.

The amino acid sequence of the opsin has been determined independently by two groups (Ovchinnikov *et al.*, 1979; Khorana *et al.*, 1979). It consists of 248 amino acid residues; of these 70% are hydrophobic and distributed in clusters. The Schiff base linkage exists between the retinal and Lys₂₁₆ of the opsin. Low resolution two

dimensional X-ray crystal structure and electron microscope studies of bR shows seven α -helices crossing the membrane almost at right angle and the molecules exist in cyclic trimers in a highly regular hexagonal lattice (Henderson and Unwin, 1975; Henderson, 1975, 1977).

The bR molecule exists in light adapted (bR^{LA}) ($\lambda_{\text{max}} = 568 \text{ nm}$) and dark adapted (bR^{DA}) ($\lambda_{\text{max}} = 558 \text{ nm}$) forms. Upon absorbing a photon, bR^{LA} undergoes a photocycle characterized by several distinct intermediate species of various colors. Protons are pumped out of the membrane during the photocycle thus establishing a proton gradient. The total number of intermediates appearing in the bR photocycle and their sequence of occurrence are still under debate. Under physiological conditions, the bR photocycle typically takes about 10 msec to be completed. On the basis of their low temperature transient absorption studies, Lozier *et al.* had proposed a scheme of the bR photocycle (Lozier *et al.*, 1975; Lozier & Niederberger, 1977) as shown in figure 1.7. According to their scheme, the primary event in the photo-absorption of bR^{LA} is the formation of an intermediate K₆₁₀ (subscript denotes the λ_{max} in nm) with a red shifted absorption. This process takes very short time (<1psec) to be completed and is the only photochemical step in bR photocycle. The intermediate K₆₁₀ undergoes dark reaction, and is converted to L₅₅₀ in about 1 μsec . The L₅₅₀ intermediate in turn decays to M₄₁₂ in about 50 μsec . The M₄₁₂ intermediate successively passes through N and O₆₄₀ intermediates finally converting back to bR^{LA} and the cycle is completed. The N intermediate was not observed but was proposed in the scheme of bR photocycle (Lozier *et al.*, 1975; Lozier & Niederberger, 1977).

Extensive Raman vibrational studies have been reported on bR and its photo-intermediates. These studies show that the chromophore in bR^{LA}

is in the all-*trans* isomeric configuration (Pettei *et al.*, 1977; Aton *et al.*, 1977). The retinal in bR^{LA} is bound to the opsin via a protonated Schiff base linkage (Lewis *et al.*, 1974). The dark-adapted form of bR (bR^{DA}) is found to contain a mixture of all-*trans* and 13-*cis* chromophores and its Schiff base linkage is also protonated (Aton *et al.*, 1979). In dark, bR^{LA} converts to bR^{DA}.

The intermediates K₆₁₀ and L₅₅₀ have been found to contain 13-*cis* retinal chromophores (figure 1.3) and the Schiff base linkages in both of them are protonated (Pande *et al.*, 1981; Braiman and Mathies, 1982; Argrade and Rothschild, 1983; Smith *et al.*, 1984). As in the case of rhodopsin, the primary process of photo-transduction in bR is the isomerization of the protonated chromophore. The difference between the primary events in photo-transduction of rhodopsin and bR is that in case of rhodopsin the isomerization is from 11-*cis* to all-*trans* (in bathorhodopsin) whereas in case of bR it is from all-*trans* to 13-*cis* in the K₆₁₀ intermediate.

Resonance Raman studies have shown that the M₄₁₂ intermediate in bR photocycle contains an unprotonated Schiff base linkage (Lewis *et al.*, 1974; Aton *et al.*, 1977; Braiman and Mathies, 1980). The chromophore in this case is also a 13-*cis* isomer. Of all the intermediates, M₄₁₂ is the only unprotonated species found in bR photocycle. This intermediate plays a crucial role in the photocycle as it has been associated with the pumping of protons in bR (Lozier *et al.*, 1975; Li *et al.*, 1984). Flash photolysis studies of bR have shown that the decay of M₄₁₂ is biphasic (see e.g. Hess and Kuschmitz, 1977; Ort and Parson, 1978). This biphasic process is sensitive to pH (Korenstein and Hess, 1982; Lam and Packer, 1983; Li *et al.*, 1984), light intensity (Ohno *et al.*, 1981; Korenstein and Hess, 1982), ionic strength (Eisenbach *et al.*, 1979) and temperature (Li *et al.*, 1984).

These observations suggest the presence of two forms of M_{412} , which decay at different rates and whose relative amounts are sensitive to the above mentioned conditions.

The N intermediate has not been observed under physiological conditions. Recently, its Raman spectrum has been reported under alkaline pH and high ionic strength conditions (Fodor *et al.*, 1988). It contains a protonated Schiff base linkage with the opsin and has a 13-*cis* retinal chromophore.

The O_{640} intermediate has been shown to contain a protonated Schiff base linkage with the opsin. Its chromophore is believed to be in a distorted all-*trans* conformation (Smith *et al.*, 1983).

The bR photocycle can be summarized as follows. First the chromophore in bR^{LA} is isomerized by light from all-*trans* to the 13-*cis* form in K_{610} intermediate. K_{610} converts to L_{550} . Then a proton is ejected out that results in the formation of the unprotonated M_{412} intermediate. M_{412} picks up a proton and converts to the N intermediate. N isomerizes from 13-*cis* to a distorted all-*trans* to produce the O_{640} intermediate. Complete isomerization of the chromophore of O_{640} intermediate to the all-*trans* conformation results in the formation of bR^{LA}.

1.4. Colors of retinal pigments

All the retinal pigments discussed above (also see Table-I) contain either 9-*cis*, 11-*cis*, 13-*cis* or all-*trans* retinal as their chromophores (figure 1.3). It is interesting to note that retinals in solution absorb near 380 nm, but when they are used as a prosthetic group in a protein the absorption shifts to higher wavelength. Pigments are seen to have colors shifted up to as high as 640 nm (as in the O_{640} intermediate of bR). Retinal Schiff bases

(SB) absorb at 360 nm in solution and protonated Schiff bases of retinal (PSB) absorb at 440 nm. This shift in absorption upon protonation can be explained by the delocalization of the π -electrons due to the added charge at the Schiff base linkage. All the pigments having λ_{max} towards the red end of the visible spectrum have protonated Schiff base chromophores (Table-I). In the absence of the surrounding opsin, these PSBs are expected to have absorption ~440 nm. The additional shift of color from 440 nm to the λ_{max} of the pigment is caused by the interactions of opsin and the chromophore and has been termed as 'opsin shift' (Nakanishi *et al.*, 1980). The value of the opsin shift may be used as a measure of the chromophore-protein perturbations in retinal pigments. Opsin shifts for some of the retinal pigments are given in Table-I.

TABLE: I

Retinal pigments, nature of their bonds with opsin and the opsin shifts. First column lists some of the retinal pigments and the second column lists their corresponding approximate absorption maxima. The third column lists the nature of the Schiff base bond between the chromophore and the opsin (or amine). The Schiff base linkages of the pigments corresponding to the empty rows are not known. The last column lists the opsin shift of the pigments with protonated Schiff base linkage with the PSB in solution taken as the reference. Opsin shifts are calculated using the following formula

$$\text{opsin shift} = \left[\frac{1}{\lambda_{\text{max of PSB in solution}}} - \frac{1}{\lambda_{\text{max of pigment}}} \right]$$

where the λ_{max} s are expressed in cm.

TABLE: I

Chromophore (Absorption center)		$\lambda_{\max}$ (nm)	Schiff base linkage	Opsin shift (cm^{-1})
Bovine	Rhodopsin	498	$-\text{C}=\text{N}^{\oplus}\text{H}-$	2647
	Bathorhodopsin	543	$-\text{C}=\text{N}^{\oplus}\text{H}-$	4311
	Lumirhodopsin	497		2607
	Meta-I	478	$-\text{C}=\text{N}^{\oplus}\text{H}-$	1807
	Meta-II	380	$-\text{C}=\text{N}^{\oplus}\text{H}-$	
Octopus	Rhodopsin	472	$-\text{C}=\text{N}^{\oplus}\text{H}-$	1541
	Bathorhodopsin	540	$-\text{C}=\text{N}^{\oplus}\text{H}-$	4209
	Lumirhodopsin	485		
	acid-meta	514	$-\text{C}=\text{N}^{\oplus}\text{H}-$	3272
	alkaline-meta	376	$-\text{C}=\text{N}-$	
Owlfly	Rhodopsin	345	?	- 6258 ?
	Bathorhodopsin	not yet observed		
	Lumirhodopsin	375		
	acid-meta	475	$-\text{C}=\text{N}^{\oplus}\text{H}-$	1675
	alkaline-meta	370		
Bacterio- rhodopsin	bR^{DA}	560	$-\text{C}=\text{N}^{\oplus}\text{H}-$	4870
	bR^{LA}	568	$-\text{C}=\text{N}^{\oplus}\text{H}-$	5122
	K_{610}	610	$-\text{C}=\text{N}^{\oplus}\text{H}-$	6334
	L_{550}	550	$-\text{C}=\text{N}^{\oplus}\text{H}-$	4545
	M_{412}	412	$-\text{C}=\text{N}-$	
	O_{640}	640	$-\text{C}=\text{N}^{\oplus}\text{H}-$	7102
In Ethanol	PSB	440	$-\text{C}=\text{N}^{\oplus}\text{H}-$	0
	SB	360	$-\text{C}=\text{N}-$	

SB: Schiff base of retinal
 PSB: Protonated Schiff base of retinal
 bR^{DA} : Dark-adapted Bacteriorhodopsin
 bR^{LA} : Light-adapted Bacteriorhodopsin

Figure 1.1 The ground and excited state electronic potential energy curves and representative vibrational modes that contribute to stokes and anti-stokes Raman scattering. The scattering processes depicted involve resonance enhancement since $h\nu_I \sim \Delta E_{eg}$.

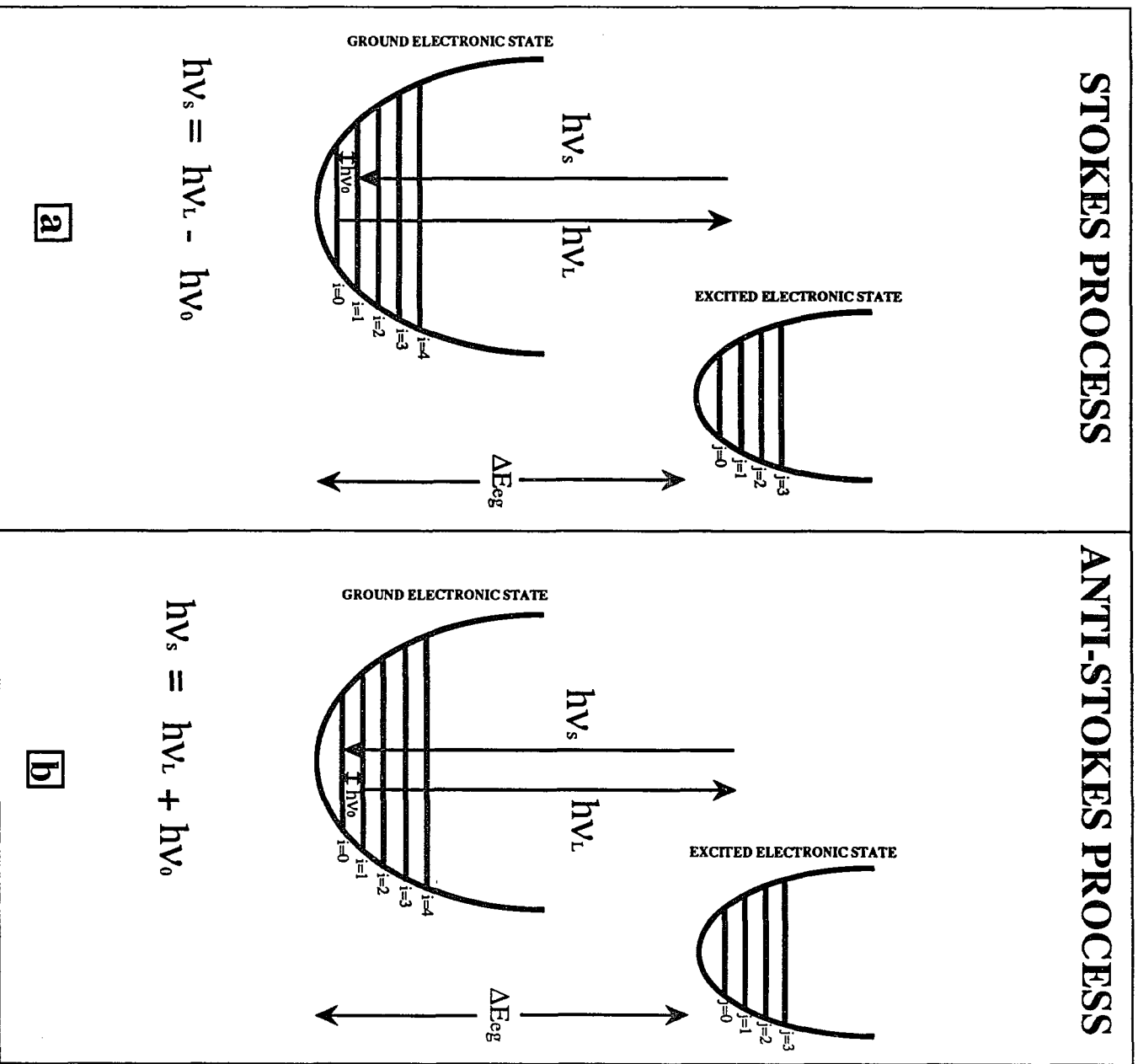


Figure 1.1

Figure 1.2 Sketch of a vertebrate retinal rod cell. Typically, a rod is $\sim 6\mu$ in diameter and $\sim 60\mu$ long containing about 2000 discs. Rhodopsin molecules are situated in these discs.

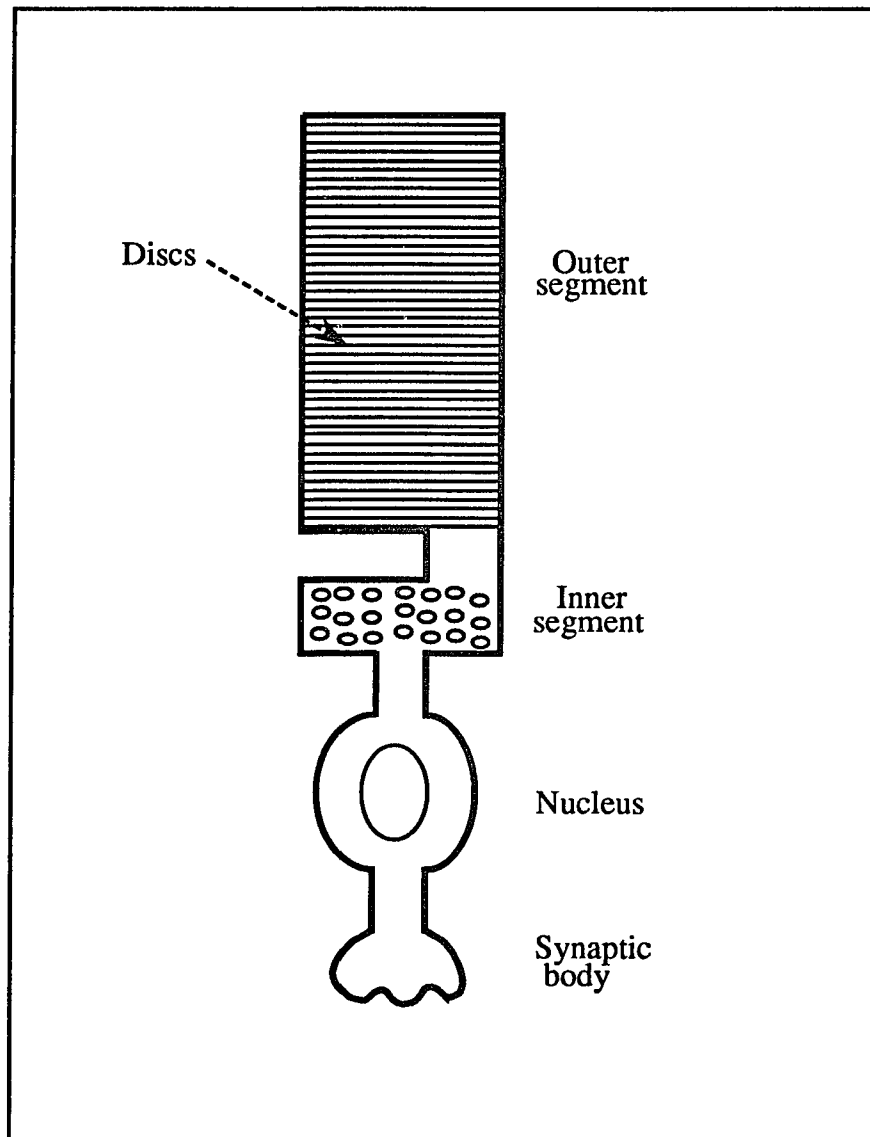


Figure 1.2

Figure 1.3 Sketches of all-*trans* (a), 13-*cis* (b) and 11-*cis* (c) retinal chromophores. R is the amino acid end group of the protein (usually the amino terminus of a Lys residue in both visual pigments and bacteriorhodopsin), responsible for the Schiff base linkage.

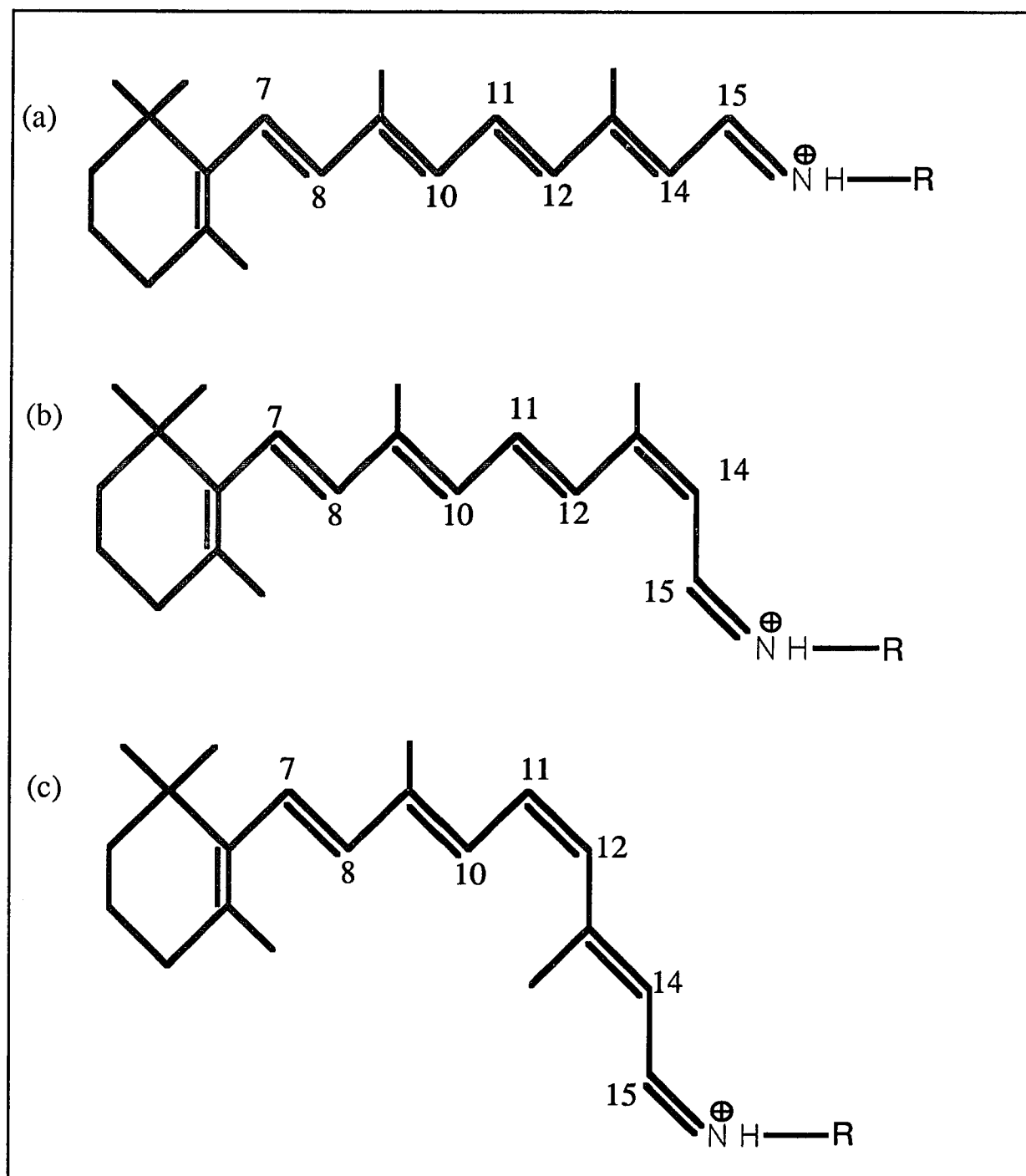


Figure 1.3

Figure 1.4 Bleaching sequence of bovine rhodopsin. The wavy lines depict photo-reactions and the solid lines represent dark-reactions. The numbers inside the parentheses are the absorption maxima of the corresponding intermediates.

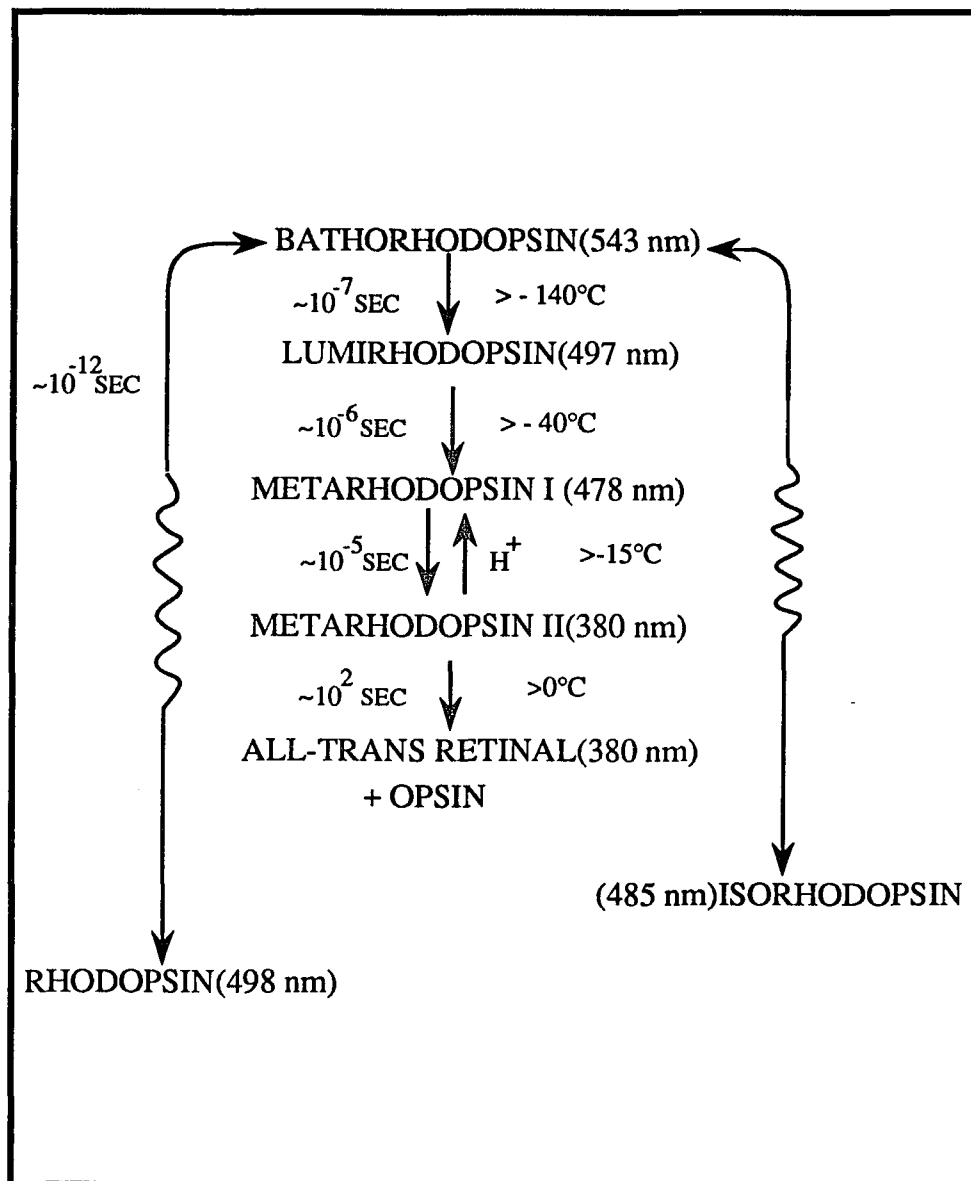


Figure 1.4

Figure 1.5 A scheme of the rhodopsin to bathorhodopsin photo-transition in the primary process of vision. The 11-*cis* chromophore of rhodopsin isomerizes to a distorted all-*trans* isomer in the bathorhodopsin. The Schiff base proton can form salt bridge with a negatively charged protein residue.

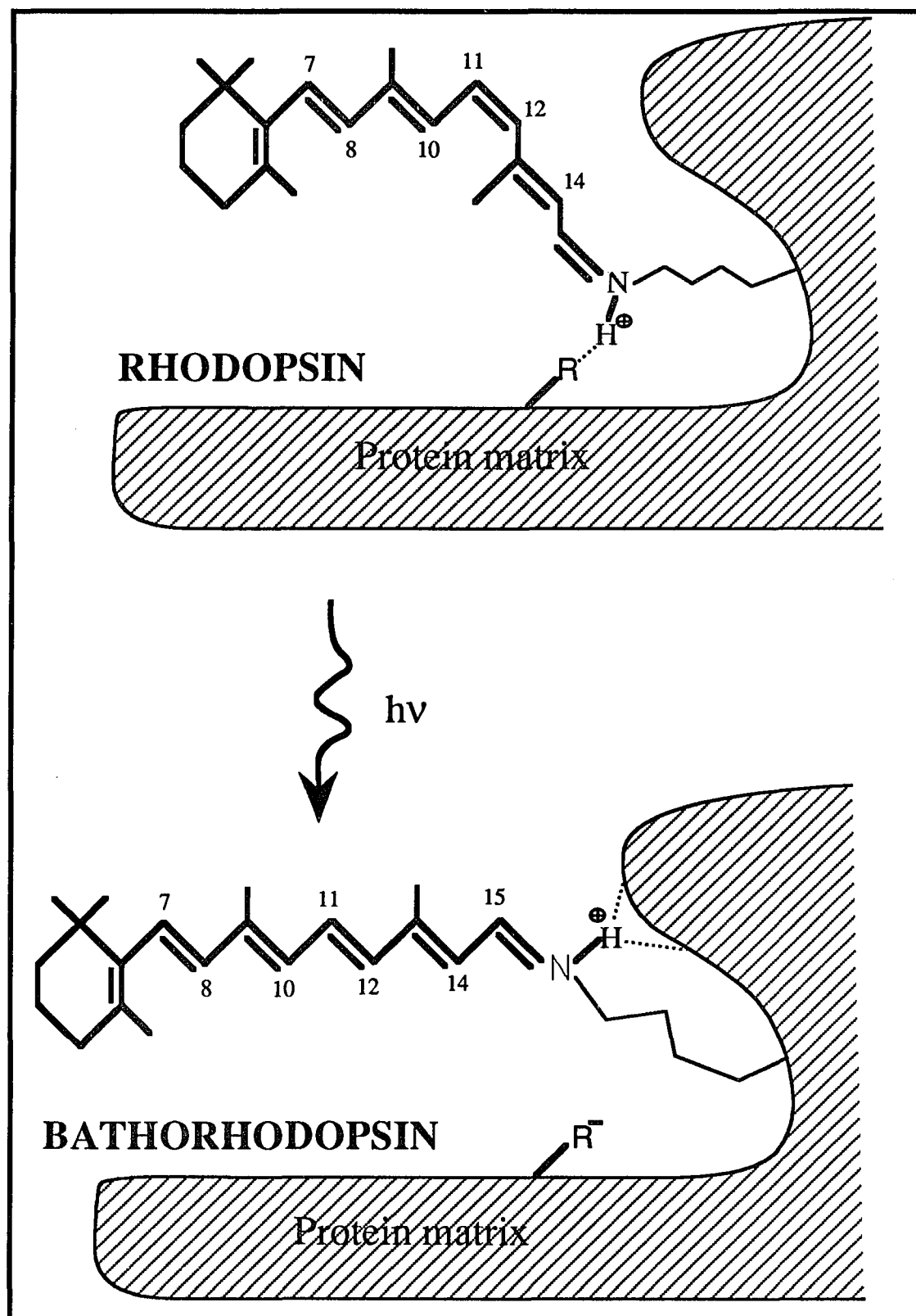


Figure 1.5

Figure 1.6 Light and dark reactions of owlfly rhodopsin. The photoreactions have been denoted by wavy lines, while the thermal (dark reactions) are represented by straight lines.

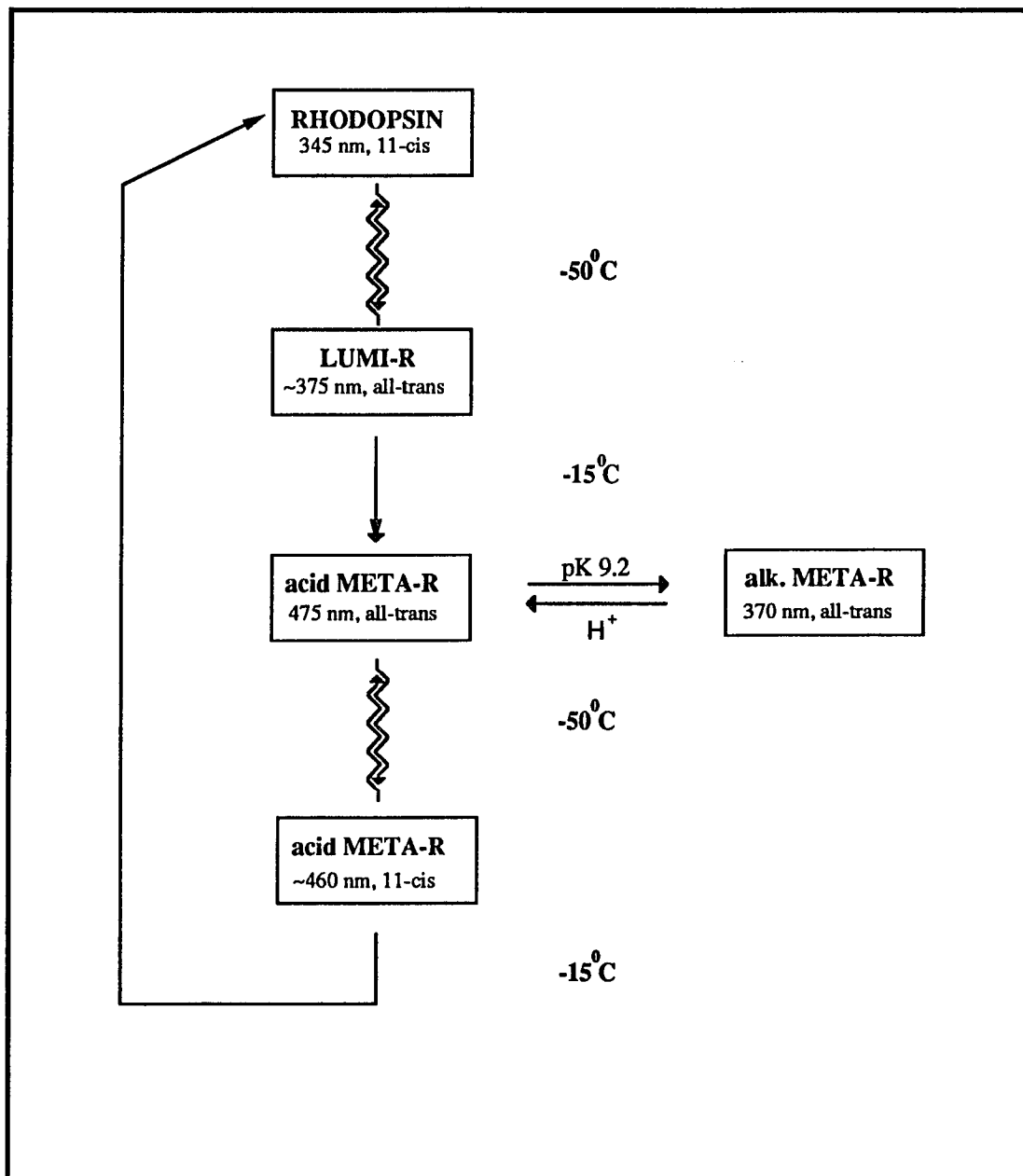


Figure 1.6

Figure 1.7 Photocycle of bacteriorhodopsin as proposed by Lozier *et al.* (1975, 1977) showing K, L, M, N and O intermediates. The subscript represents the approximate absorption maximum of the corresponding intermediate. Solid arrows depict dark-reactions and wavy arrows depict photo-reactions. The N intermediate was not observed until recently (Fodor *et al.*, 1988; Kouyama *et al.*, 1988).

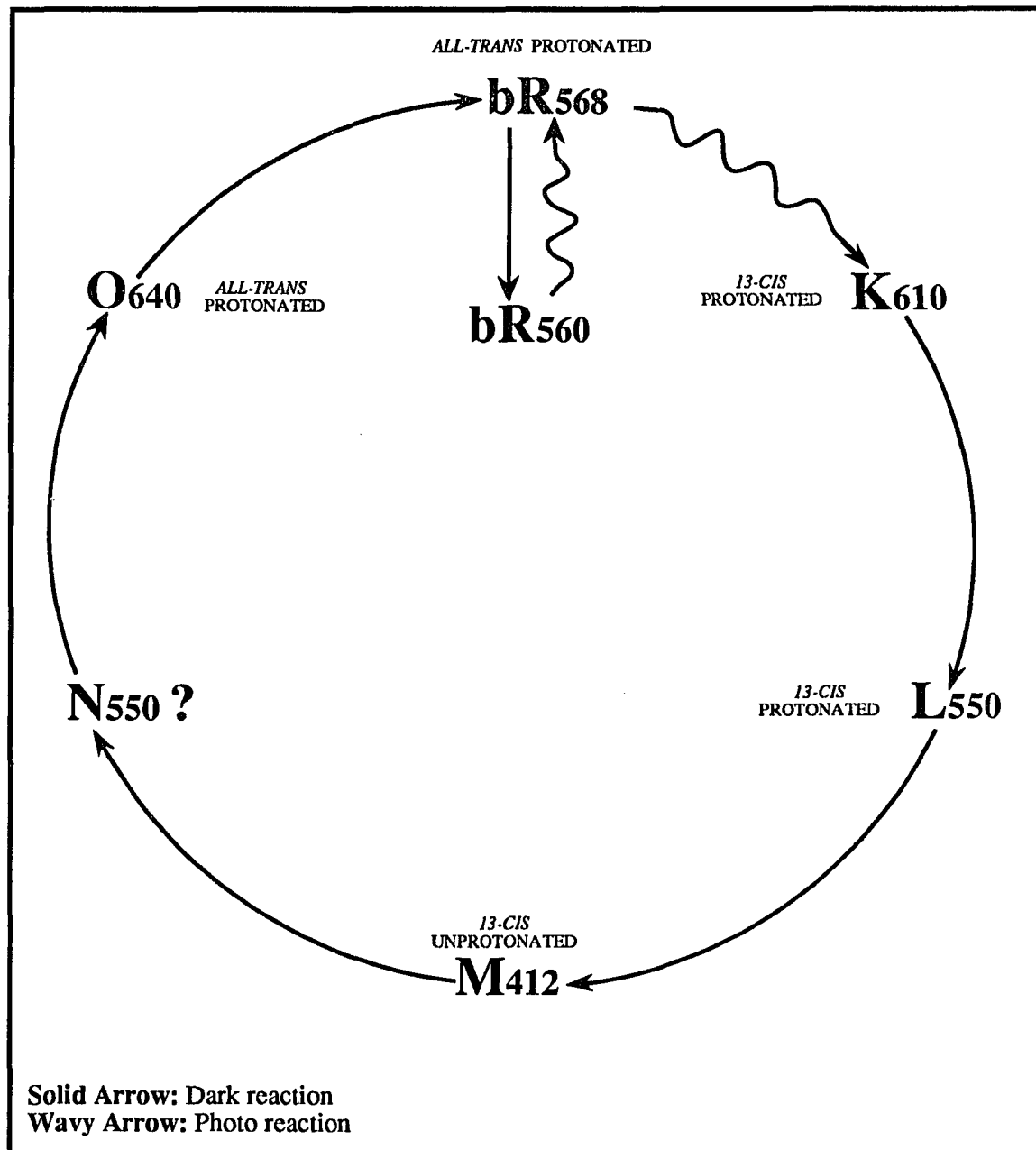


Figure 1.7

CHAPTER 2

Experimental

2.1. Instrumentation

For absorption measurements of the sample, a Perkin-Elmer model Lambda-3b spectrophotometer was used which is interfaced to a Macintosh personal computer. Data were stored and analyzed in the computer. The sample holder of the spectrophotometer was modified and was connected to a Lauda circulator bath (Model RM-6) to achieve temperature control of the sample. The bath temperature could be varied in the range from -15°C to $+120^{\circ}\text{C}$ with an accuracy of 0.1°C using ethylene glycol and water in proper proportion as the circulating fluid. Condensation in the sample chamber was avoided by flowing dry air through it.

Raman spectra were obtained with one or the other of the following spectrometers. One of the spectrometer is a double monochromator (Model 1401, Spex industries) attached to a cooled RCA model 31034 photomultiplier tube and a photo counting device interfaced to a LSI-11 minicomputer (Plessey peripherals). The double monochromator was controlled by the minicomputer via a CD2A control board (Spex industries). Although this spectrometer-photomultiplier combination is slow in operation, the RCA photomultiplier (model 31034) tube had a better response in the red region of the visible spectrum and the spectral resolution is better than the triplemate spectrometer (see below). The second spectrometer consists of a Spex triplemate (Model 1877, Spex industries) and a optical multichannel analyzer system (OMA) having a reticon photodiode array (Model 1420-2, EG&G Princeton Applied Research) with a thermo-electric cooling device and a multichannel detector

controller (Model 1218, Princeton Applied Research). The detector temperature can be locked at -20°C . The triplemate-OMA combination is interfaced to a LSI-11 computer for faster operation and operated by the software developed in our laboratory. The multichannel system had 700 active pixels for data collection capable of displaying a window of size up to 1400 cm^{-1} , depending on the turret grating in use (either of the 600, 1200, 1800 groves/cm grating). The triplemate spectrometer was set up on the top of a vibrationless optical table. The spectral resolution was typically between 5 and 7 cm^{-1} depending on the laser excitation.

For the Raman excitation, either a krypton ion laser (Model CR-2000, Coherent) or an argon ion laser (Model 165, Spectra-Physics) was used. Same lasers were used for optical pumping of the sample. For the light adaptation of bacteriorhodopsin a Kodak 250W slide projector with Corning glass filter (3-70) was used. The same slide projector was also used for bleaching octopus rhodopsin. A Sorval RC2-B centrifuge with a SS-34 rotor was used for preparing concentrated samples.

2.2. Room temperature Raman setup

For Raman measurements of the samples at temperatures from room temperature down to 0°C a home made cell holder connected to a Lauda circulator bath was used. Typically, sample was taken in a $3\times 3\text{ mm}$ quartz cuvette and placed in this cell holder. Sample was excited by a laser line hitting the bottom of the cuvette vertically upwards and the scattered light was collected at right angles by a $f=1.2$ camera lens. The collected light was focussed on the entrance slit of the spectrometer.

2.3. Low temperature Raman setup

The low temperature Raman spectra were obtained using a home made nitrogen cold finger dewar connected to a flexible nitrogen/helium transfer line (Model LT-3-110, Air Products and Chemicals, Allentown, PA). The sample was transferred onto a concave depression (40 μ l volume) of a copper block (copper tip) and mounted on a temperature controlled copper sample holder. The sample holder was connected to the other end of the nitrogen/helium transfer line and was placed inside a home made glass jacket. Helium gas was flowed through the jacket to avoid condensation of air at low temperatures. The copper tip was arranged for right angle scattering from the surface of the sample. The temperature of the sample holder could be controlled between 85K to room temperature using liquid nitrogen and was monitored using a platinum thermistor temperature sensor.

2.4. Flow setup

Sample flow was necessary in situations where the photo excited molecules must be removed from the laser beam (e.g. in the experiment with bR at pH 10, see chapter 6). The flow setup consisted of a home made 100 ml capacity conical double layered glass sample reservoir. Arrangement was made for magnetic stirring of the sample in the reservoir. The outer jacket of the reservoir can be connected to a Lauda circulator bath to control the temperature of the sample. The outlet at the bottom of the conical sample reservoir was connected to a 0.5 mm i.d. glass capillary by a 2 mm i.d. tygon tubing. The other end of the capillary was connected to a peristaltic pump and then to the sample reservoir. The flow speed of the sample can be controlled and a maximum speed of 750 cm/sec inside the capillary can be achieved.

2.5. Spinning cell setup

Spinning cell setup is an ideal arrangement to obtain Raman spectra of various photo intermediates of bacteriorhodopsin. In this setup a 5 cm diameter and 0.5 cm chamber thickness plexy glass circular cell is used to spin the sample at high speed. The shaft of the cell is connected to an electrical motor. This cell can be spun up to a speed of 2700 rpm. This setup was used for the experiments with 13-bromo-bR.

2.6. Data acquisition and manipulation

Absorption data were stored in a Macintosh personal computer and manipulated by a program developed in our laboratory. Raman spectra are stored in LSI-11 computers. The double monochromator is calibrated with the known frequency of the laser line. The data from 700 pixels of the OMA is calibrated against the known Raman lines of toluene spectrum. The accuracy of the peak position is different for each experiment and is in the range of 2-3 cm^{-1} . The resolution of the spectra are wavelength dependent and typically it is $\sim 7 \text{ cm}^{-1}$ at 500 nm from the triplement. Because of slight difference in the amplification between odd and even pixels of the photodiode array, a simple two point averaging is performed (when needed) to obtain a smooth curve. Dark current from the reticon system is not a problem for short time signal averaging but it gives a major contribution to the background when the signal averaging time is several seconds. In such a situation, dark current is recorded for the same length of signal integration time as for the real spectra and is subtracted out of the Raman spectrum before frequency calibration.

CHAPTER 3

Study of the bR₄₅₀ intermediate of Bacteriorhodopsin regeneration

3.1. Introduction

The various colors of retinal pigments are believed to be governed mainly by two factors: the protonation state of the retinal-protein linkage and the spatial arrangement of the charged residues in the protein matrix surrounding the chromophore (see e.g. Honig and Ebrey, 1982). In an effort to understand the nature of the protein-chromophore interactions several low absorbing retinal pigments have been reported. (1) A ca. 480 nm absorbing species (bR₄₈₀) has been observed when bR is solubilized in detergents (Konishi & Packer, 1977; London & Khorana, 1982) and this seems to contain a protonated Schiff base linkage between the chromophore and the protein (Pande *et al.*, 1989a). (2) At alkaline pH (pH > 11.5), bR is converted to an unprotonated species absorbing maximally at ~460 nm (bR₄₆₀) (Druckmann *et al.*, 1982). (3) Delipidation of bR yields a new species absorbing maximally at ~480 nm (bR₄₈₀) which has been shown to contain a protonated Schiff base linkage (Baribeau and Boucher, 1985; Pande *et al.*, 1989b). Dark reconstitution of bR is possible from bR-opsin and either 13-*cis* or all-*trans* retinal (Oesterhelt & Schuhmann, 1974). Oesterhelt and co-workers (Oesterhelt & Schuhmann, 1974) had observed two intermediates of bR-regeneration occurring sequentially. The earlier intermediate (bR₄₀₀) was reported to have absorption maximum at 400 nm and 440 nm for the later. Our computer subtraction data show that this later intermediate absorbs maximally at ~450 nm (see section 3.3), so we choose

to call this the bR₄₅₀ intermediate. On the basis of similarities between reactions of opsin with either retinal or retinol, the chromophore in bR₄₀₀ was suggested to be bound to the opsin non-covalently. The chromophore in the later intermediate does not react with the reducing agent NaBH₄ (Schreckenbach *et al.*, 1977,1978).

In order to determine the nature of chromophore-protein linkage in the bR₄₅₀ intermediate of bacteriorhodopsin regeneration and to explore the mechanism of regeneration we have regenerated bR in both water and deuterium oxide and studied this intermediate by resonance Raman spectroscopy. Our results show that there is a protonated schiff base linkage between the chromophore and the protein and suggest that the color shift of the all-*trans* retinal from 380 nm in solution to 570 nm in regenerated bR is achieved through several steps in the regeneration process (see discussion below).

3.2. Materials and methods

Apo-membrane (bR opsin) was a generous gift from the laboratory of Prof. T.G. Ebrey at the University of Illinois, Urbana. This was stored at 4°C until further use. A Perkin-Elmer Lambda 3B spectrophotometer was used for absorption measurements. The sample holder was modified to allow temperature control by connecting to a refrigerated circulator bath (Lauda). For regeneration experiments the temperature of the sample holder was maintained at 5°C. One ml of bR-opsin having an absorbance of 1 OD at 280 nm was taken in a one cm path quartz cuvette (Hellma) and 3 µl of all-*trans* retinal in ethyl alcohol (total amount equivalent to an absorbance of 0.2 OD at 380 nm in 1 ml volume) was added to the opsin followed by mixing. Absorption scans were taken without any reference at a rate of 120

nm/min at suitable intervals, and were stored in a computer. Opsin spectrum was computer subtracted to obtain pure spectra of the regenerated intermediate(s).

For Raman measurements, 500 μ l of opsin with a typical absorbance of 8 OD at 280 nm was pelleted by centrifuging at 13000 rpm for 2.5 hrs in a Sorval RC2-B centrifuge (SS-34 rotor) to a final volume of 50 μ l. Amount of all-*trans* retinal needed for complete regeneration was determined from a separate titration experiment. Five microliters of retinal solution in ethyl alcohol (equivalent of 0.75 OD at 380 nm when diluted to 1ml), just enough for complete reaction, was added to the pellet and mixed using a pipette at $\sim 10^{\circ}\text{C}$ temperature. The mixture was then transferred to a copper tip of the low temperature setup described in chapter 2 (section 2.3). Sample was cooled to 90K temperature in ~ 10 min and this temperature was maintained throughout the experiment. The 465.7 nm line from an argon ion laser (Spectra Physics, model 165) was used for Raman excitation. Raman scattered light was collected at 90° to the exciting beam and passed through a triplemate spectrometer (Spex industries, Model 1877) and reticon photo-diode array system (Princeton Applied Research, Model 1420) at a resolution of 7 cm^{-1} . Data were collected for three hours and stored in a LSI-11 minicomputer (Plessey peripherals) for correction of reticon response and the spectra were calibrated against the standard peaks of a toluene spectrum.

For the measurements in D_2O , the bR-opsin was pelleted by centrifuging at 13000 rpm for 2.5 hrs (Sorval RC2-B centrifuge, SS-34 rotor), supernatant discarded and the pellet dissolved in D_2O (99.8% D, Sigma). This was pelleted again and the process was repeated two more

times. The pellet was finally dissolved in D₂O. The retinal solution for these measurements was prepared in deuterated ethyl alcohol.

3.3. Results

3.3a. Absorption

The spectra of the regenerated preparation at 5°C temperature are shown in figures 3.1 (H₂O) and 3.2 (D₂O). Opsin spectra were computer subtracted to obtain the spectra showing only the chromophoric absorption. Spectra 3 through 8 in figure 3.1 (regeneration in H₂O) pass through a common isosbestic point at 484 nm whereas spectrum 2 passes very close to it. Spectrum 1 in this figure does not pass through the isosbestic point of others and has higher absorbance on the low wavelength side. This could be due to the fact that spectrum 1 (also spectrum 2) is a mixture of bR₄₀₀ and bR₄₅₀ intermediates. Similarly, spectra 1 & 2 of figure 3.2 (regeneration in D₂O) contain contributions from bR₄₀₀ besides the bR₄₅₀ species. The isosbestic point in D₂O is at 477 nm. The single isosbestic point observed both in water and D₂O suggests that bR₄₅₀ decays directly to bR. The regenerated bR has $\lambda_{\max}$ at ~570 nm which implies that light-adapted form of bR (bR^{LA} or bR₅₇₀) is obtained in the regeneration from all-*trans* retinal. Spectra rich in either bR₄₀₀ or bR₄₅₀ intermediate with little or no contribution of bR was obtained from spectra 1, 3 and 7 of figure 3.1 by appropriate subtraction that removes the contribution from bR and are shown in figure 3.3. The bR₄₅₀ chromophore (in H₂O) has $\lambda_{\max}$ at 453 nm (figure 3.3b, curve 2) and the bR₄₀₀ intermediate has $\lambda_{\max}$ at 423 nm (figure 3.3b, curve 1). Instead of having a single uniform absorption band, both bR₄₀₀ and bR₄₅₀ are seen to have several shoulders to their main absorption bands (multi-peaked absorption). It is seen from figures 3.1 and

3.2 that most of the bR_{400} species decay to the bR_{450} intermediate within the first 5 min (at 5°C) whereas appreciable amount of bR_{450} species are present even after 30 min of regeneration. The regeneration of bR is slower in D_2O than in H_2O . This is realized when the ratios of absorbances at 570 nm and 450 nm of the sample in H_2O are compared with that in D_2O [e.g. after 40 min of regeneration $\Delta A_{570}/\Delta A_{450} = 2.17$ for H_2O and 1.09 for D_2O sample].

3.3b. Resonance Raman

3.3b.1. Ethylenic region

Resonance Raman spectra of bR_{450} in normal H_2O and D_2O are shown in figure 3.4 and 3.5 respectively. The most intense band from the $C=C$ stretch appears at $\sim 1568\text{ cm}^{-1}$ in both water and D_2O . The shoulder around 1530 cm^{-1} in both the water and deuterated spectra is due to some contribution from bR, already formed from bR_{450} . This contribution is quite small to make any appreciable change in the finger print region of the intermediate. No major contribution from the unreacted retinal to the Raman spectra is expected for the reason that the amount of retinal added is just sufficient for complete reaction with the opsin (see materials and methods) and our absorption results show that the retinal-protein reaction is completed in 5 min at room temperature.

3.3b.2. Schiff base region

The Schiff base vibration ($C=N$ stretch) shows an isotope effect as it shifts downward in frequency when the nitrogen is deuterated. Comparison of the figures 3.4 and 3.5 shows that the Schiff base frequency at 1667 cm^{-1} in water shifts to 1631 cm^{-1} in the deuterated

sample. This proves that the Schiff base linkage in bR₄₅₀ is protonated. The presence of a protonated Schiff base linkage further suggests that the species we are looking at is bR₄₅₀ and not bR₄₀₀ because the latter was shown to be bound non-covalently to the opsin (Schreckenbach *et al.*, 1977), thus should have no observed Schiff base mode. The position and the amount of downward shift of the Schiff base frequency upon deuteration is sensitive to the environment of this bond. The shift of more than 35 cm⁻¹ observed in this case is much larger compared to the 17 cm⁻¹ shift for bR^{LA} (see e.g. Pande *et al.*, 1981) and could be due to a stronger hydrogen bonding interaction between the Schiff base proton and the protein in the bR₄₅₀ intermediate. The chromophore-protein interaction must be very different than that exists in native bR. The positions of the Schiff base peak in both water and deuterated bR₄₅₀ are very close to the frequency observed for the corresponding protonated Schiff base of retinal (PSB) in solution. This suggests that except for the protein counter-ion near the Schiff base other protein charges do not affect the Schiff base frequency significantly.

3.3b.3. Fingerprint region

The fingerprint region (1100-1350 cm⁻¹) of the Raman spectra of retinal pigments contain mostly C--C (single bond) stretch and C--C--H bending mode frequencies (Callender and Honig, 1977) and is sensitive to the isomeric configuration of the chromophore as well as to the nature of protein-chromophore interactions. This region of bR₄₅₀ Raman spectrum is similar to, but not the same as, that of both 13-*cis* and all-*trans* PSB in solution (Aton *et al.*, 1977; Doukas *et al.*, 1978; Pande *et al.*, 1981). For example, the three major lines at 1169, 1202 and 1280 cm⁻¹ of bR₄₅₀ have

their counterparts at 1164, 1198, 1277 cm^{-1} in the spectrum of all-*trans* PSB and at 1175, 1203, 1271 cm^{-1} in the spectrum of 13-*cis* PSB in solution. The 1202 cm^{-1} band of bR₄₅₀ is broad and might have a shoulder on the low frequency side (not marked). Although these observations do not lead to a conclusive answer as to whether the chromophore of bR₄₅₀ is all-*trans* or 13-*cis*, comparison of the fingerprint region of bR₄₅₀ with that of the all-*trans* chromophore of bR^{LA} and the 13-*cis* chromophore of bR₅₄₈ (Smith *et al.*, 1984) suggests that the chromophore in bR₄₅₀ is essentially all-*trans*.

3.4. Discussion

It was suggested by Oesterhelt and co-workers (Schreckenbach *et al.*, 1977) that the 400-nm intermediate in bR regeneration is non-covalently bound to opsin, more like retinal in the active binding site of opsin, and this results in a color shift from 380 nm to 400 nm. Our Raman results show that bR₄₅₀ is bound to the opsin via a protonated Schiff base linkage. This probably accounts for the next phase of color shift, i.e. from 400 nm to 450 nm. Although we were unable to determine the rate of formation and decay of bR₄₀₀ due to instrumental limitations, our absorption measurements suggests a value of $t_{1/2} \ll 5\text{min}$ for the decay of bR₄₀₀. Conversion of bR₄₅₀ to bR is very slow ($t_{1/2} \sim 20\text{ min}$ at 5°C), and accounts for the final phase of color shift, i.e. from 450 nm to 570 nm. In the light of these observations it can be said that when retinal is added to opsin it diffuses to the opsin active site quickly (in a diffusion time scale) forming a chromophore absorbing at $\sim 400\text{ nm}$. The next phase of reaction is the establishment of a protonated Schiff base linkage with the protein

which shifts the absorption to 450 nm. In the final step the opsin folds around the chromophore and the regeneration of bR is complete. Since the Schiff base frequency of bR₄₅₀ is very close to the one observed for PSB in solution, it can be said that the effect of opsin on the Schiff base bond of bR₄₅₀ is equivalent to that of chloride ions or water dipoles on PSB in solution.

The differences in the Schiff base region between bR₄₅₀ and bR^{LA} can be accounted for by the fact that upon rearrangement (folding), opsin places additional charged residues around this bond to perturb its frequency and decrease the hydrogen bonding nature of the Schiff base. A possible rearrangement of the Schiff base bond itself upon protein folding cannot be ruled out. The differences observed in the fingerprint region could also be due to the interactions between the charged amino acid residues of the protein and the chromophore itself. The multi-peaked absorption curves seen in both bR₄₀₀ and bR₄₅₀ (figure 3.3) are very similar to those observed for retro-retinal chromophores. This has led to the suggestion that the retinal is first planarized before fitting in to the opsin active site (Schreckenbach *et al.*, 1977) but there was no direct evidence of chromophore planarization in these intermediates. Our Raman data of the bR₄₅₀ intermediate are not sufficient to suggest planarization of the ionone-ring.

The difference between bR₄₆₀ (Druckmann *et al.*, 1982) and the intermediate bR₄₅₀ is that the former is an unprotonated Schiff base species and is strongly influenced by the surrounding opsin which results in the color shift from 360 nm to 460 nm for an unprotonated chromophore whereas in the latter case this influence seems to be minimal and it resembles more like *trans*-PSB in solution. Either delipidation or

solubilization of bR in DMSO gives rise to bR₄₈₀ species. These species are protonated Schiff base bound chromophores where the protein has rearranged its structure resulting in a decrease in protein-chromophore interactions that causes the hypsochromic color shift (Pande et al, 1989a, 1989b). The bR₄₅₀ species seems to be an extreme case of bR₄₈₀, where the chromophore-protein interactions are almost absent outside the Schiff base region. This minimal protein perturbations outside the Schiff base region has also been observed in the 9-*cis* isorhodopsin of the 440 nm absorbing visual pigment of *Bufo marinus* (Loppnow *et al.*, 1989) where the Schiff base mode appears at 1662 cm⁻¹ and shifts down to 1636 cm⁻¹ upon deuteration.

Figure 3.1 Regeneration of bacteriorhodopsin in H₂O from all-*trans* retinal and opsin at 5°C temperature. No reference was used. Spectra presented here were obtained after subtracting the opsin absorption. Scans were recorded at a speed of 120nm/min starting at (1) 1min, (2) 6min, (3) 10min, (4) 14.5min, (5) 19.7min, (6) 24min, (7) 30min and (8) 40min after adding retinal to the opsin.

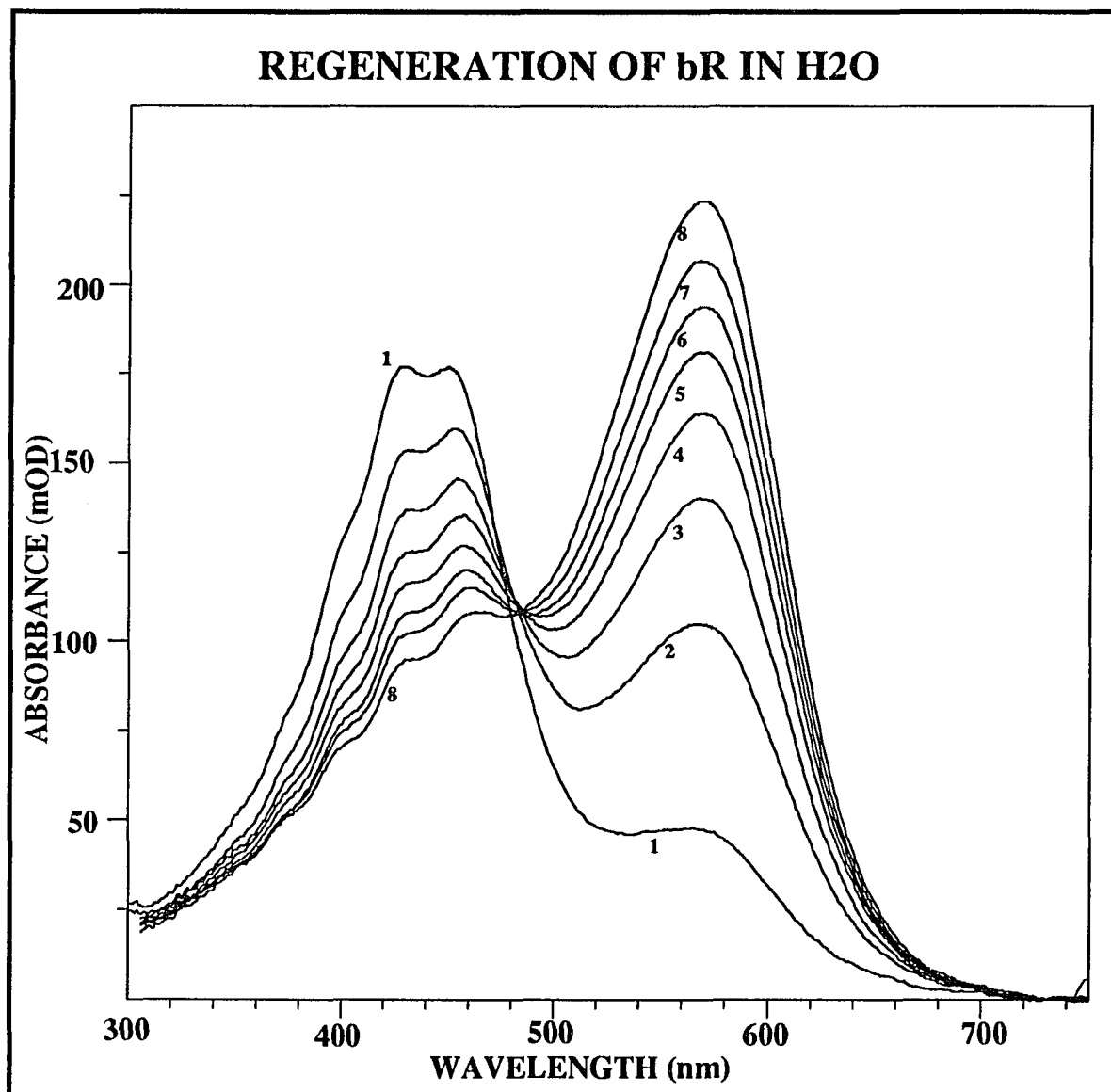


Figure 3.1

Figure 3.2 Regeneration of bacteriorhodopsin in D₂O from all-*trans* retinal and opsin at 5°C temperature. No reference was used. Spectra presented here were obtained after subtracting the opsin absorption. Scans were recorded at a speed of 120nm/min starting at (1) 0.5min, (2) 5min, (3) 9.8min, (4) 14.5min, (5) 19.15min, (6) 23.8min, (7) 28.5min, (8) 40min, (9) 50min and (10) 60min after adding retinal to the opsin.

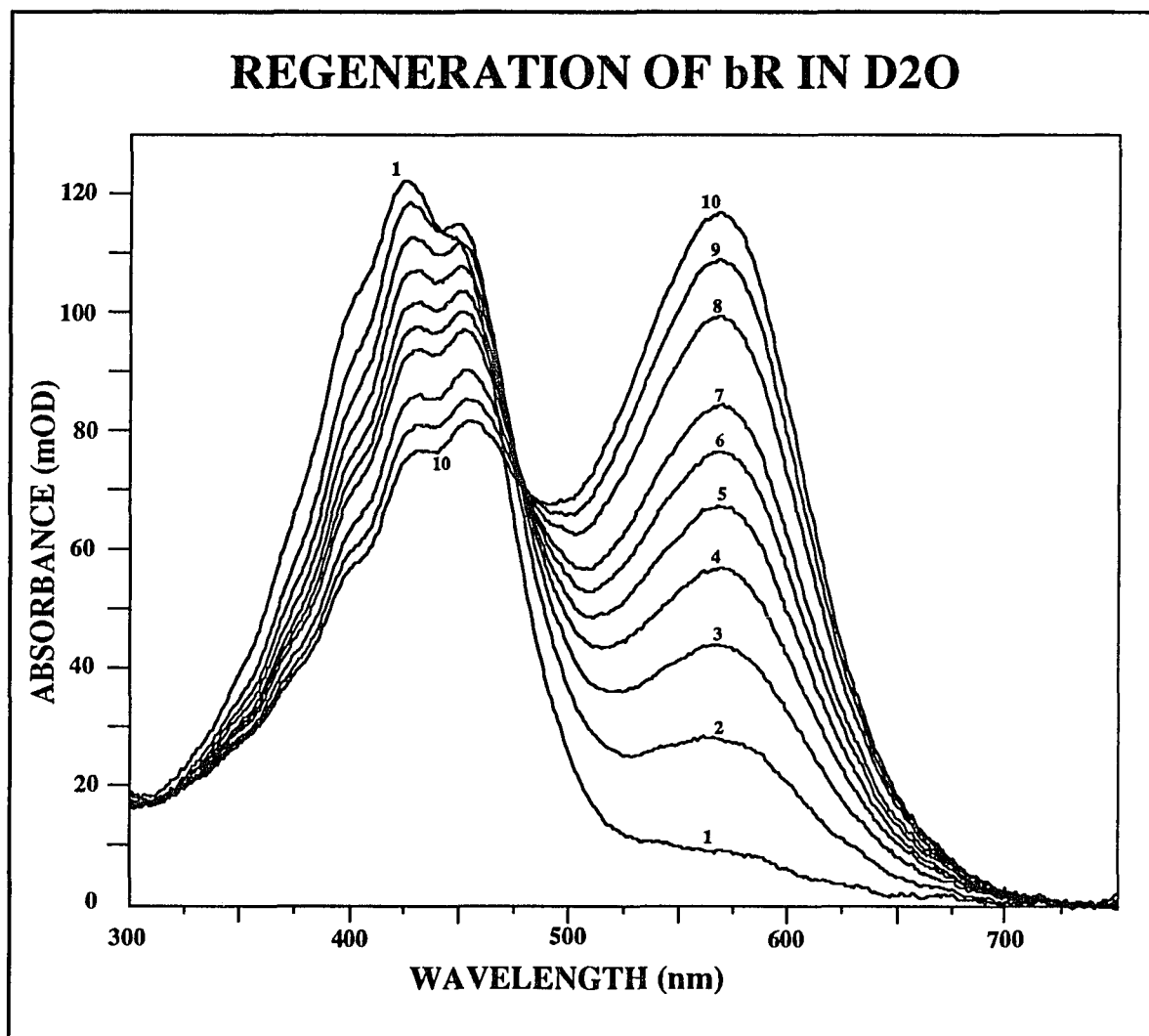


Figure 3.2

Figure 3.3 bR_{400} and bR_{450} intermediate species in H_2O . The curves 1&2 of figure 3.3a were obtained after subtracting the spectrum 7(30min) from spectra 1(1min) and 3(10min) of fig.3.1, respectively, until contribution of bR_{570} was completely removed. Curve 3.3b 1(2) was obtained by subtracting arbitrary amount of 3.3a 2(1) from 3.3a 1(2) and represents the bR_{400} (bR_{450}) intermediate only.

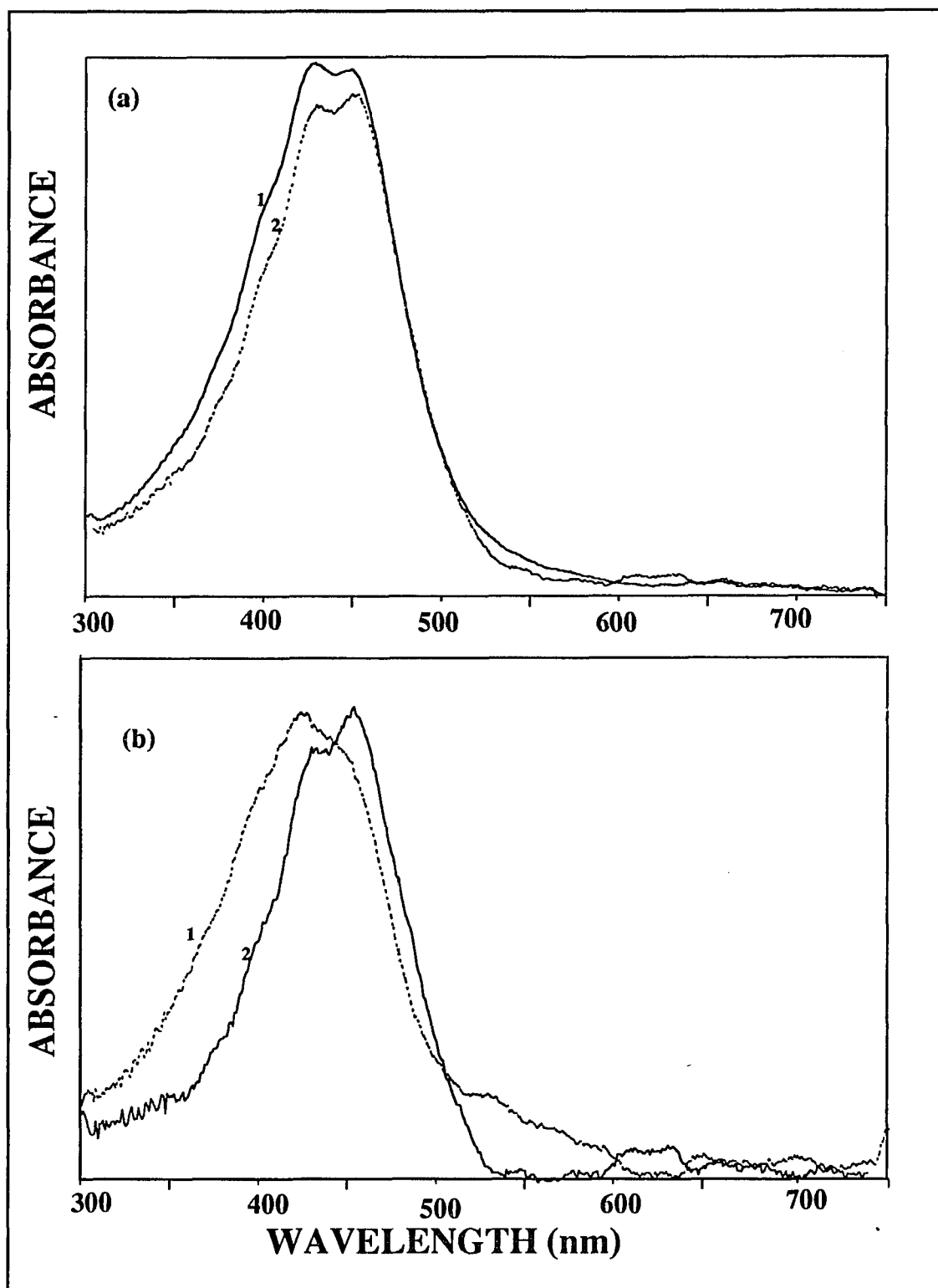


Figure 3.3

Figure 3.4 Low temperature (90K) Raman spectrum of the bR₄₅₀ intermediate of bacteriorhodopsin regeneration in H₂O. The 465.7 nm beam of an argon ion laser was used for Raman excitation.

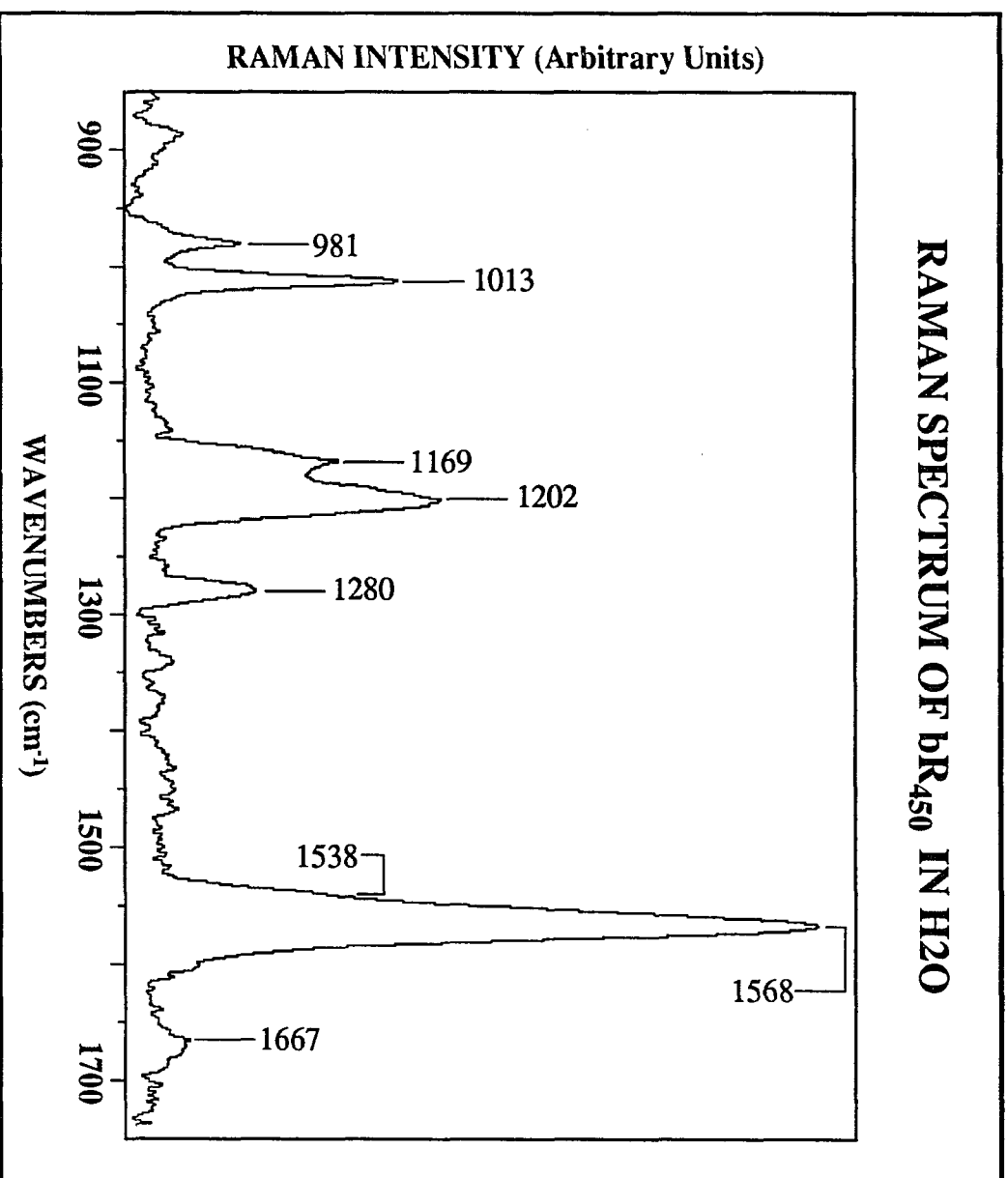


Figure 3.4

Figure 3.5 Low temperature (90K) Raman spectrum of the bR₄₅₀ intermediate of bacteriorhodopsin regeneration in D₂O. The 465.7 nm beam of an argon ion laser was used for Raman excitation.

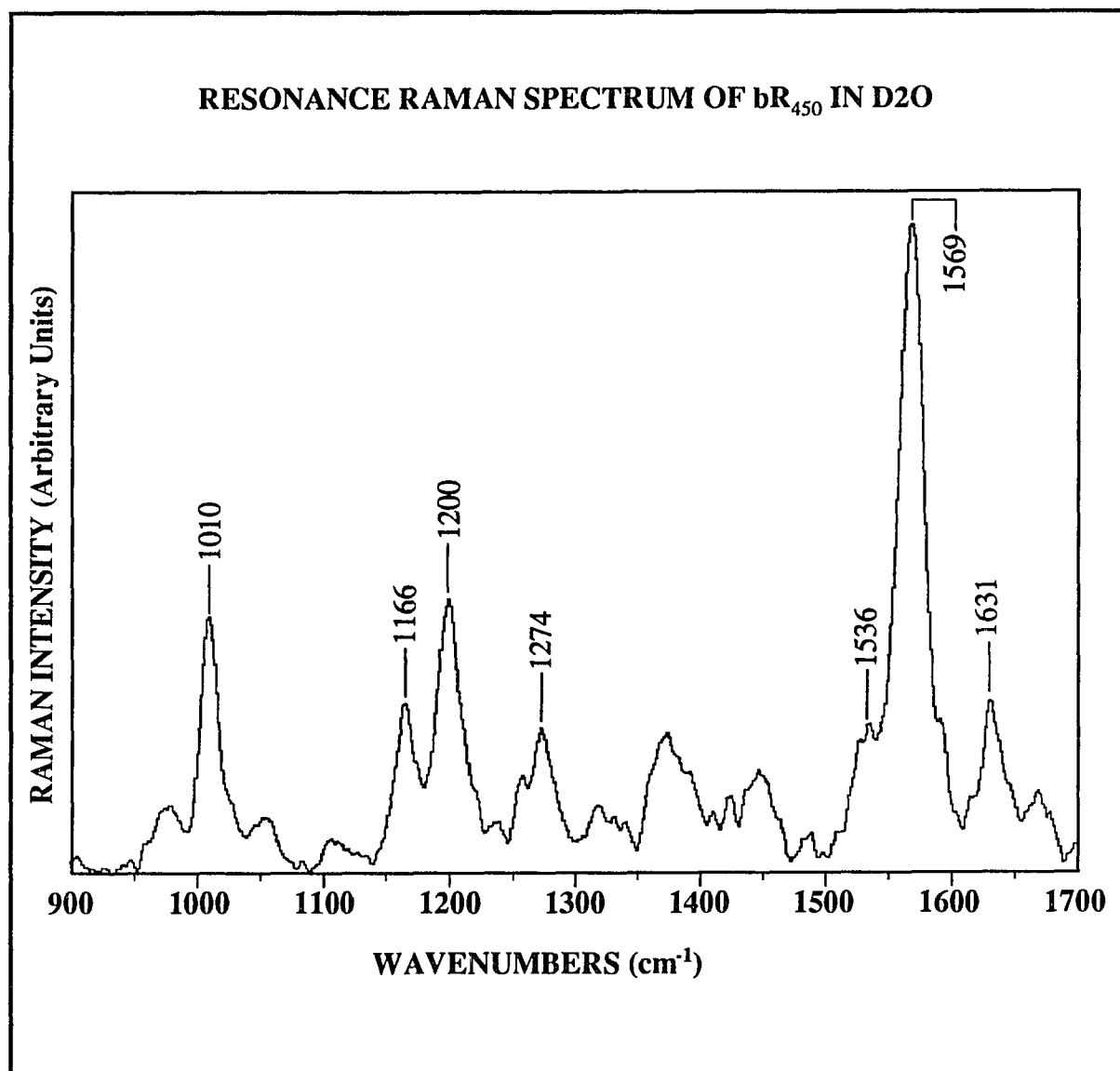


Figure 3.5

CHAPTER 4

Study of the UV sensitive visual pigment of *Ascalaphus macaronius*

4.1. Introduction

The bovine visual pigment rhodopsin is a 38,000 dalton retinal protein containing 11-*cis* retinal linked to the surrounding protein via a protonated Schiff base linkage (Hubbard and St. George, 1958; Oseroff and Callender, 1974). Upon absorption of a photon rhodopsin ($\lambda_{\max}$ = 498 nm) is converted to a pigment with red shifted absorption (bathorhodopsin, $\lambda_{\max}$ ~ 543 nm) (Yoshizawa & Wald, 1963) (figure 1.5). Bathorhodopsin contains an all-*trans* chromophore linked to the opsin via a protonated Schiff base linkage. At physiological temperatures bathorhodopsin undergoes thermal (dark) reactions and finally bleaches to retinal and protein (Wald, 1968). The bleaching sequence is shown in figure 1.4. Calorimetric studies have shown that ~32 kcal/mol of energy is stored during the rhodopsin to bathorhodopsin transition (Cooper *et al.*, 1979; Schick *et al.*, 1987). This translates to about 67% of the incident photon energy. This primary step in vision, i.e. rhodopsin to bathorhodopsin transition, is an important step since the energy captured in the isomerization reaction of this step is believed to be later utilized for the excitation of retinal rod cells and thus completing vision.

Absorption of a photon by octopus rhodopsin ($\lambda_{\max}$ = 472 nm) results in the formation of bathorhodopsin ($\lambda_{\max}$ = 540 nm) just as in the case of vertebrate bovine rhodopsin. Octopus bathorhodopsin then undergoes

series of dark reactions resulting in acid (alkaline) metathodopsin. Chemical extraction and resonance Raman studies (Pande *et al.*, 1987) show that octopus rhodopsin contains a 11-*cis* protonated Schiff base chromophore whereas its bathorhodopsin has an all-*trans* chromophore (still protonated), similar to their bovine counterpart. Calorimetric studies have shown that about 32 kcal/mol of incident photon energy is captured during the rhodopsin to bathorhodopsin transition of octopus visual pigment (Cooper *et al.*, 1986). Again we notice here that in the case of octopus visual pigment, the primary step of vision is the isomerization of the chromophore (11-*cis* to all-*trans*) accompanied by the photon energy capture.

Bacteriorhodopsin (bR) ($\lambda_{\text{max}} = 568\text{nm}$) is a light energy transducing retinal-protein system which supports the energy requirements of the microorganism *Halobacterium halobium* under low oxygen supply. The chromophore in the light-adapted bR is a protonated all-*trans* retinal Schiff base which undergoes isomerization to the 13-*cis* isomer (K_{610} intermediate of bR photo-cycle, see section 1.3 of chapter 1) upon absorption of a photon (figure 1.7). This results in the storage of ~16 kcal/mol of the incident photon energy in the K_{610} intermediate (Birge and Cooper, 1983). This is another example of light energy transducing system where the primary step of energy transduction is the isomerization (in this case all-*trans* to 13-*cis*) of a protonated Schiff base chromophore.

In all the light energy transducing systems described above a protonated (charged) Schiff base chromophore is a common feature. The step in the energy transduction process in which isomerization of this charged chromophore inside the protein cavity occurs is the step where the

incident photon energy is captured. These observations have laid the foundations of the models for the process of energy transduction in retinal based pigments (Honig *et al.*, 1979). Theoretical calculations were able to predict a substantial amount of the total absorbed photon energy by the spatial motion of the charged (protonated) chromophore in the electrostatic environment of the surrounding protein (Honig *et al.*, 1979; Deng and Callender, 1987; Birge *et al.*, 1988; Rodman Gilson *et al.*, 1988). These models require that the chromophore in the pigment be protonated and there must be isomerization of this chromophore to capture the incident photon energy.

Protonation of the chromophore seems to be a key requirement in the model for visual transduction process. Retinals in solution absorb near 380 nm and retinal Schiff bases absorb at ~360 nm. Protonation of the Schiff base results in a red shifted absorption from 360 nm to ~440 nm. On the basis of studies on model compounds, this bathochromic shift is believed to arise from an increased π -electron delocalization caused by the presence of a proton in the Schiff base bond. The additional red shift observed in the protonated Schiff base chromophores of bovine rhodopsin ($\lambda_{\text{max}} = 498$ nm), octopus rhodopsin ($\lambda_{\text{max}} = 472$ nm) and bR^{LA} ($\lambda_{\text{max}} = 568$ nm) is thought to be caused by the electrostatic interactions of the chromophore with the surrounding protein, probably involving some charged groups (see e.g. Honig and Ebrey, 1982), and is termed opsin shift. All the light energy transducing systems described above possess a protonated chromophore and have positive opsin shift ($\lambda_{\text{max}} > 440$ nm). Our goal here is to extend these observations and test the model for visual transduction in the visual pigment of *Ascalaphus macaronius*.

The rhodopsin of *Ascalaphus macaronius* absorbs maximally at ~345 nm and was shown to contain 11-*cis* retinal chromophore (Paulsen and Schwemer, 1972), like that of bovine, squid and octopus rhodopsins. This brings at least two issues in to consideration. First, since the $\lambda_{\max}$ of owlfly rhodopsin is much closer to the $\lambda_{\max}$ of unprotonated Schiff base in solution than that of protonated Schiff base, what is the nature of chromophore-opsin linkage (protonated or unprotonated) in this system? Secondly, if it does have a protonated Schiff base chromophore, like other systems studied so far, what is so different in the chromophore-protein interaction that causes a large blue shift ($\lambda_{\max} < 440$ nm) instead of a conventional red shift ($\lambda_{\max} > 440$ nm) observed for bovine, squid and octopus rhodopsins?

Owlfly rhodopsin undergoes a series of dark reactions upon irradiation, as shown in figure 1.6. Absorption of photons converts rhodopsin to the supposed (yet unobserved) primary photoproduct, bathorhodopsin, which then thermally decays to metarhodopsin via lumirhodopsin. Unlike bovine metarhodopsin which is unstable and hydrolyzes to retinal and opsin, the metarhodopsin of owlfly as well as other invertebrates (squid and octopus) is stable and can be photochemically converted back to the primary pigment, rhodopsin quantitatively (for example, see figure 4.1).

Raman spectra of the owlfly acid metarhodopsin and some preliminary results of its rhodopsin are presented here. The data show that the owlfly acid metarhodopsin contains a protonated Schiff base linkage between the chromophore and the opsin just like its counterpart in bovine and octopus visual system. Rhodopsin data strongly favors a protonated

chromophore-opsin Schiff base linkage. These data suggest that the protonated Schiff base linkage between the protein and the chromophore may be a property that is shared by all the species thereby meaning a general mechanism of visual transduction. In addition, there are marked similarities among the Raman spectra of the acid metarhodopsins of owlfly and octopus, bovine metarhodopsin I, and the protonated Schiff bases of all-*trans* model compounds in solution. This strongly suggests that owlfly acid metarhodopsin contains an all-*trans* retinal isomer.

4.2. Materials and methods

Retinas were isolated from *Ascalaphus* (owlfly) eyes, collected and homogenized in phosphate buffer (0.1M, pH=6.0). Following centrifugation, the pellet was resuspended in 35% sucrose (w/v; in phosphate buffer), layered under phosphate buffer, and centrifuged again. Rhabdomeric membranes were collected from the interface and washed several times with phosphate buffer. After the final centrifugation, the pellets were irradiated with blue light (472 nm interference filter) in order to convert possible fractions of acid metarhodopsin to rhodopsin and stored at -80°C until further use. This portion of the sample preparation was performed in the laboratory of Prof. J. Schwemer at Ruhr-Universität Bochum, FRG.

For the measurements at 10°C, the rhodopsin was solubilized in 15 mM CHAPSO (Calbiochem). For deuteration experiments, the CHAPSO solution was prepared in D₂O, and the rhodopsin was deuterated by spinning down twice in D₂O prior to solubilization. The sample in CHAPSO was sonicated for 15 minutes and was then centrifuged at 10,000

rpm for 10 minutes to remove the insoluble matter. The supernatant was used for both the Raman and absorption measurements.

The absorption spectra were measured in a Perkin-Elmer Lambda 3b spectrophotometer using quartz microcuvettes (volume 20 μ l, Hellma). The typical rhodopsin extracts used for Raman measurements had an absorbance of 1 OD at 345 nm.

The Raman spectra of acid metarhodopsin were obtained at 10°C from a detergent solubilized sample contained in a 3x3mm quartz cuvette in a thermostated cell holder which was cooled by a Lauda circulator bath. The rhodopsin spectra were obtained at -100°C from an unsolubilized sample contained in the cold finger of the low temperature setup described earlier (see section 2.3 of chapter 2). The cuvette setup typically required a 40 μ l sample while the low temperature setup needed a 30 μ l sample volume. Due to its red shifted absorption (λ_{max} = 475 nm) compared to rhodopsin, the acid metarhodopsin spectra were obtained with 457.9 nm line from an argon ion laser (Spectra Physics, Model 165). The rhodopsin (λ_{max} = 345 nm) spectra were obtained by exciting the sample with 406.7 nm line from a krypton ion laser (Coherent, Model CR-2000). The 350.7 nm line from the krypton ion laser was also tried for rhodopsin but was unsuitable due to enormous sample fluorescence. All the Raman spectra were stored in a mini computer for further analysis. It should be mentioned here that we tried various wavelengths for the pump and probe beams to obtain Raman signal from either rhodopsin or acid metarhodopsin of owl, but except for those mentioned above all the rest of them were found to be unsuitable. For example, at -190°C temperature we tried 350, 406.7, 467.5, and 457.9 nm probe excitations in absence of a pump beam.

We also tried both 350 and 413.1 nm probes along with a simultaneous 488 nm pump beam, but only sample fluorescence was observed. At -100°C we tried 350, 406.7 and 413.1 nm excitations which resulted in either enormous fluorescence or the signals from sample impurities.

4.3. Results and Discussion

4.3a. Absorption

Figure 4.1 shows the absorption spectra of *Ascalaphus* rhodopsin. The small tailing contribution in the visible region to the absorption spectrum is due to some contaminants [cytochrome(s) and/or accessory pigments] present in a small amount. Prolonged exposure of this extract to the light of 350 nm (xenon arc 150W, interference filter) converts a maximal fraction of approximately 70% to acid metarhodopsin (curve 2, figure 4.1). This in turn can be completely converted back to rhodopsin by blue light irradiation (e.g. 472 nm). It should be mentioned that while similar photoreversibility is known to exist in all invertebrate visual pigments studied so far (Stavenga & Schwemer, 1984), the vertebrate visual pigments hydrolyze to retinal and opsin upon irradiation. Like in cephalopods, the acid metarhodopsin of owl is converted to an alkaline form upon raising pH ($pK = 9.2$). This has prompted the suggestion that the binding of the chromophore to the opsin in acid metarhodopsin occurs via a protonated Schiff base linkage which deprotonates upon the formation of alkaline metarhodopsin (Schwemer *et al.*, 1971).

4.3b. Resonance Raman

The H₂O and D₂O Raman spectra of acid metarhodopsin are shown in

figure 4.2a and 2b respectively. The spectra were obtained using the 457.9 nm probe beam from an argon ion laser (15 mW), while a coaxial 350.7 nm beam (200 mw) was pumping the rhodopsin formed by the probe beam back to acid metarhodopsin (see figure 4.1). The details of the spectra are discussed below.

4.3b.1. Ethylenic region

The dominant, Raman active, ethylenic band (C==C stretch) of acid metarhodopsin appears at 1556 cm^{-1} (figure 4.2a). It has been observed that the position of the ethylenic frequency is inversely correlated to the absorption maximum of the pigment (Aton *et al.*, 1977; Doukas *et al.*, 1978). One such correlation plot is shown in figure 4.3. The acid metarhodopsin absorption maximum at 475 nm would suggest an ethylenic band at 1555 cm^{-1} , in excellent agreement with the observed position. There is also a marked shoulder at the lower frequency side of the strong 1556 cm^{-1} band. This presumably arises from the other intermediate(s) which precede the acid meta, in the process of photoconverting rhodopsin to acid metarhodopsin, e.g., the bathorhodopsin. The observed correlation between the ethylenic stretching frequencies and the absorption maxima of visual pigments and bacteriorhodopsin would suggest that the absorption maximum of this (these) species be red shifted compared to the acid metarhodopsin. The predominant band, however, is clearly due to acid metarhodopsin and, therefore, it is reasonable to believe that the other spectral features are representative of the acid metarhodopsin (also see below). These spectra were collected in 1 hr and by this time the intensity of the Raman signal from the sample had decreased appreciably,

presumably due to the sample bleaching under these light intensities.

While measuring the spectra in D_2O , shown in figure 4.2b, we slightly modified the procedure to avoid contributions from the other intermediate(s). Instead of using simultaneous pump probe technique, we first illuminated the sample with the pump beam, converting the sample to acid metarhodopsin, blocked the pump beam, and then collected the Raman scattering for ~ 5 min with the probe beam. During this time the acid metarhodopsin spectrum was observed to progressively decrease in intensity due to its re-conversion to rhodopsin. This whole procedure was repeated several times and the data collected for ~ 1 hr. As can be seen, although the ethylenic peak position appears at the same position (1555 cm^{-1}) as in figure 4.2a, the shoulder observed in figure 4.2a has almost disappeared and the ethylenic band is a lot sharper.

4.3b.2. Schiff base Region

The Schiff base ($C=N$) stretching mode in the acid metarhodopsin appears at 1660 cm^{-1} as seen in spectrum 4.2a. Deuteration of the sample results in the shift of this mode to 1631 cm^{-1} . These data clearly show that the Schiff base linkage in owlfly acid metarhodopsin is protonated. For comparison, the Schiff base vibrational mode in bovine metarhodopsin I and octopus acid metarhodopsin occur at 1657 and 1656 cm^{-1} , respectively (Doukas *et al.*, 1978; Kitagawa & Tsuda, 1980; Pande *et al.*, 1987). This mode was shown to shift to 1625 cm^{-1} upon deuteration (Kitagawa & Tsuda, 1980) for the octopus acid metarhodopsin.

4.3b.3. Fingerprint Region

The fingerprint region ($1100\text{-}1350\text{ cm}^{-1}$) mainly originates from C--

C stretch and the C--C--H bending motions (Callender & Honig, 1977) and is quite sensitive to the isomeric state of the chromophore, as well as to the chromophore terminal end group and, perhaps, other factors such as the nature of chromophore-protein interactions. The owlfly acid metarhodopsin spectrum (figure 4.2a) shows a predominant band at 1196 cm^{-1} in this region and two, relatively less intense, bands at 1230 and 1283 cm^{-1} . The octopus acid metarhodopsin spectrum (Pande *et al.*, 1987), for comparison, shows remarkable similarity to this spectrum with peaks at 1197 , 1227 and 1274 cm^{-1} . The general features for the metarhodopsin I intermediate of bovine rhodopsin (Doukas *et al.*, 1978) in this region, though similar, show slightly different peak positions. It is interesting to note that all these metarhodopsin species show marked similarity to the all-*trans* retinal protonated Schiff base of model compounds and are quite different from the other isomers. These data, in complete agreement with the earlier suggestions (Hamdorf *et al.*, 1973) reveal that, like in the bovine and octopus systems, the acid metarhodopsin of owlfly contains an all-*trans* chromophore. The significance, if any, of the minor differences in this region of the spectra between the acid metarhodopsin spectrum in water and in deuterated samples (figures 4.2a & 4.2b) will have to await further studies with selectively isotopically labelled retinals.

We tried various experimental configurations in order to obtain the spectrum of the primary pigment rhodopsin. Unfortunately, as mentioned above, the samples were gradually bleached under our illumination conditions. The following conditions yielded the best results. Unsolubilized rhabdomeric membranes were used and measurements were made at -100°C to maximize the amount of rhodopsin by blocking its thermal (dark) decay pathway (figure 1.6). At this temperature, the

metarhodopsins cannot be formed (Hamdorf *et al.*, 1973; Stavenga & Schwemer, 1987) and even the formation of lumirhodopsin is significantly reduced. Although we would have preferred to make the measurements at liquid nitrogen temperature (-190°C), as has been the practice in previous Raman studies of vertebrate and invertebrate rhodopsins, the overwhelming fluorescence at that temperature proved prohibitive. The 406.7 nm line from a krypton ion laser was used to selectively enhance the Raman signal from rhodopsin ($\lambda_{\text{max}} = 345$ nm). As mentioned above, all attempts to measure the scattering with 350 nm light resulted in enormous fluorescence background. It should be noted that in the same run, but with 457.9 nm light, we could not obtain any Raman signal. Unfortunately, spectra obtained with the 406.7 nm light had a significant contribution from small contaminants in the sample due to cytochrome(s) which, although do not scatter at 457.9 nm (insert to figure 4.2), show significant Raman scattering due to resonance enhancement at 406.7 nm. Raman measurements with 406.7 nm at 10°C served as the necessary control for the above experiment, since other data showed Raman scattering from the impurities but not from rhodopsin or its stable photoproduct, acid metarhodopsin, as judged by the absence of significant Raman bands in both the ethylenic and the Schiff base regions ($1550\text{-}1670\text{ cm}^{-1}$). This is not surprising, since illumination of rhodopsin ($\lambda_{\text{max}} = 345$ nm) at this temperature with 406.7 nm light will produce acid metarhodopsin ($\lambda_{\text{max}} = 475$ nm), and the Raman scattering from acid metarhodopsin is not expected to be resonance enhanced at this excitation wavelength (see materials and methods also).

Insert to figure 4.2 shows the spectral features in the $1500 - 1700\text{ cm}^{-1}$ region in the data obtained with 406.7 nm excitation at -100°C . There appears to be a small amount of contamination from the acid

metarhodopsin, as seen by the presence of a small ethylenic mode at 1555 cm^{-1} . This band is very small compared to the main band at 1605 cm^{-1} , so we expect negligible contribution to the Schiff base region from acid metarhodopsin. The shift towards the higher frequency of the ethylenic mode of rhodopsin ($\lambda_{\text{max}} = 345 \text{ nm}$) at 1605 cm^{-1} , compared to that of acid metarhodopsin ($\lambda_{\text{max}} = 475 \text{ nm}$) at 1555 cm^{-1} , is consistent with the empirically observed inverse correlation between the λ_{max} and ethylenic mode frequency for retinal based pigments (Aton *et al.*, 1977; Doukas *et al.*, 1978; also see figure 4.3). However, a quantitative extrapolation of the previously derived correlation (Doukas *et al.*, 1978) would predict a value of $\sim 1585 \text{ cm}^{-1}$ for the ethylenic mode of rhodopsin using the absorption maximum of 345 nm. It should be pointed out that the correlation is not theoretically well understood. Thus, the quantitative discrepancy in this study is quite interesting and may be useful to further the theoretical understanding of this empirical relationship.

The most interesting result of this experiment is the observed position of the Schiff base mode at 1664 cm^{-1} , close to the position of protonated Schiff base modes observed in a large variety of pigments. For example, in the owlfly acid metarhodopsin spectrum this mode appears at 1660 cm^{-1} , and in the bovine and octopus rhodopsins it appears at 1657 and 1656 cm^{-1} , respectively (see e.g., Pande *et al.*, 1987). This frequency is quite far from the observed frequencies of the unprotonated Schiff base bands which are found to lie in the region of 1620-1635 cm^{-1} . This suggests that the owlfly rhodopsin contains a protonated Schiff base linkage between the retinal chromophore and the opsin. Unfortunately, all the samples bleached before we could obtain a rhodopsin spectrum in D_2O . A deuteration effect, like that observed for the owlfly acid metarhodopsin

would be considered a definite proof.

4.4. Conclusions

There are two central attributes that characterize the primary photochemistry of visual pigments in higher animals. The first is that the retinal chromophore undergoes an 11-*cis* to all-*trans* isomerization upon the absorption of a photon. The previous results of Paulsen and Schwemer (1972) showing that owlfly contains an 11-*cis* chromophore and the present resonance Raman results suggesting that the acid metarhodopsin form contains an all-*trans* chromophore shows that this attribute extends to the insect visual pigments.

The second chemical characteristic of visual pigments is that the chromophore is linked to the protein via a protonated Schiff base. Our Raman measurements on the owlfly visual pigments show that the Schiff base linkage between the chromophore and the protein in acid metarhodopsin is protonated and strongly suggest that it is protonated in rhodopsin as well. We discuss below the important implications of a protonated Schiff base for rhodopsin, but caution that the definitive proof of the state of protonation must await a deuteration experiment as discussed above.

That the primary pigment rhodopsin is protonated is particularly remarkable since it would seem far easier for nature to achieve ultraviolet sensitivity by choosing an unprotonated Schiff base. Protonated Schiff bases of retinal in solution absorb near 440 nm while unprotonated Schiff bases absorb near 360 nm. This suggests that the chromophore is charged for some special reason. The reason would probably have to do with the conversion of light energy to chemical energy which occurs in visual

transduction. It has been proposed (Honig *et al.*, 1979b; Deng & Callender, 1987; Rodman Gilson *et al.*, 1988) that much of this energy conversion is achieved upon photoisomerization of the chromophore which results in the movement, through space, of the positively charged protonated Schiff base moiety against an electrostatic field due to opsin at the retinal binding site. The simplest imaginable mechanism involves the breaking of a salt-bridge between the protonated Schiff base of rhodopsin and a negatively charged counter ion due to the surrounding protein. Since the apoprotein (opsin) structure of rhodopsin is unknown, it is unclear how precisely the chromophore interacts with the surrounding protein. However, the positively charged chromophore is certainly electrostatically stabilized. Simple electrostatic considerations (Honig *et al.*, 1979b; Deng & Callender, 1987; Rodman Gilson *et al.*, 1988) show that considerable chemical energy can be obtained when this stabilization is disrupted by the chromophore photoisomerization. Thus, it would appear that nature has selected a common mechanism of visual transduction across the diverse species which have been studied to date.

Experimental and theoretical work (Honig *et al.*, 1979a) suggest that color regulation in visual pigments is achieved through chromophore-protein electrostatic interaction. The specific $\lambda_{\max}$ of retinal based pigments is sometimes quantified by the difference between the $\lambda_{\max}$ of a retinal protonated Schiff base in solution (440 nm) and the $\lambda_{\max}$ of the retinal pigments expressed in wavenumber (energy) units. This is the so called 'opsin shift' which must result from specific chromophore-protein interactions (also see chapter 1, section 1.4). For example, the opsin shift in bovine rhodopsin ($\lambda_{\max}$ = 498 nm) is +2647 cm^{-1} while that in

bacteriorhodopsin ($\lambda_{\text{max}} = 568 \text{ nm}$) is $+5122 \text{ cm}^{-1}$ (see Table-I). On the same basis, one observes an opsin shift of -6258 cm^{-1} (note the negative sign) in the case of owlfly rhodopsin. The specific models for color (λ_{max}) regulation in bovine rhodopsin (e.g., Honig *et al.*, 1979a) and bacteriorhodopsin (e.g., Nakanishi *et al.*, 1980; Harbison *et al.*, 1985) are based on the placement of strategically located charges or dipoles along the polyene chain of retinal. This substantially modifies the π -electron configuration and can result in significant red shifts. Qualitatively, it seems plausible that similar placement of charges (or dipoles), either in different positions and/or of opposite sign, could result in the significant blue shifts observed in UV absorbing pigments like the one present in owlfly.

Figure 4.1 Spectral absorbance of owlfly rhodopsin (spectrum 1) and acid metarhodopsin (spectrum 2). UV light (350 nm, interference filter, Xenon arc 150W) was used to set up the photo-steady-state between rhodopsin and metarhodopsin (30:70; curve 2). The inset shows the reconversion of acid metarhodopsin to rhodopsin by successive irradiation with blue light(472 nm, interference filter; curves 3,4 and 5). Rhodopsin was extracted in 50 ml of 3% CHAPSO (pH 6.0) from membranes isolated from 20 retinas.

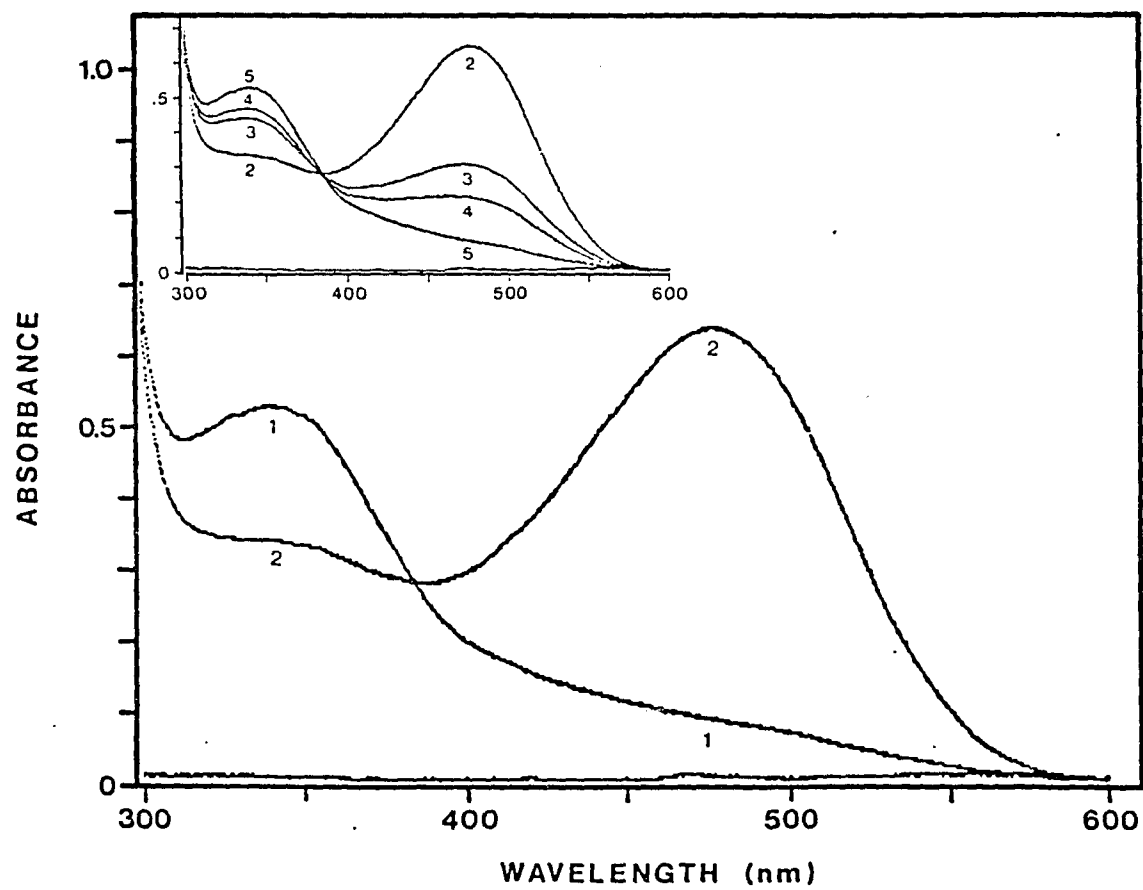


Figure 4.1

Figure 4.2 (a) Resonance Raman spectrum of *Ascalaphus* (owlfly) acid metarhodopsin in 15 mM CHAPSO at 10°C. (b) The same as (a) except the sample was deuterated. The insert shows the resonance Raman spectrum of owlfly rhodopsin at -100°C from an unsolubilized sample (not in detergents). The acid metarhodopsin spectra were measured with 457.9 nm light from an argon ion laser, while the rhodopsin spectrum was measured with 406.7 nm light from a krypton ion laser. The sample concentration was ~1 OD at 345 nm.

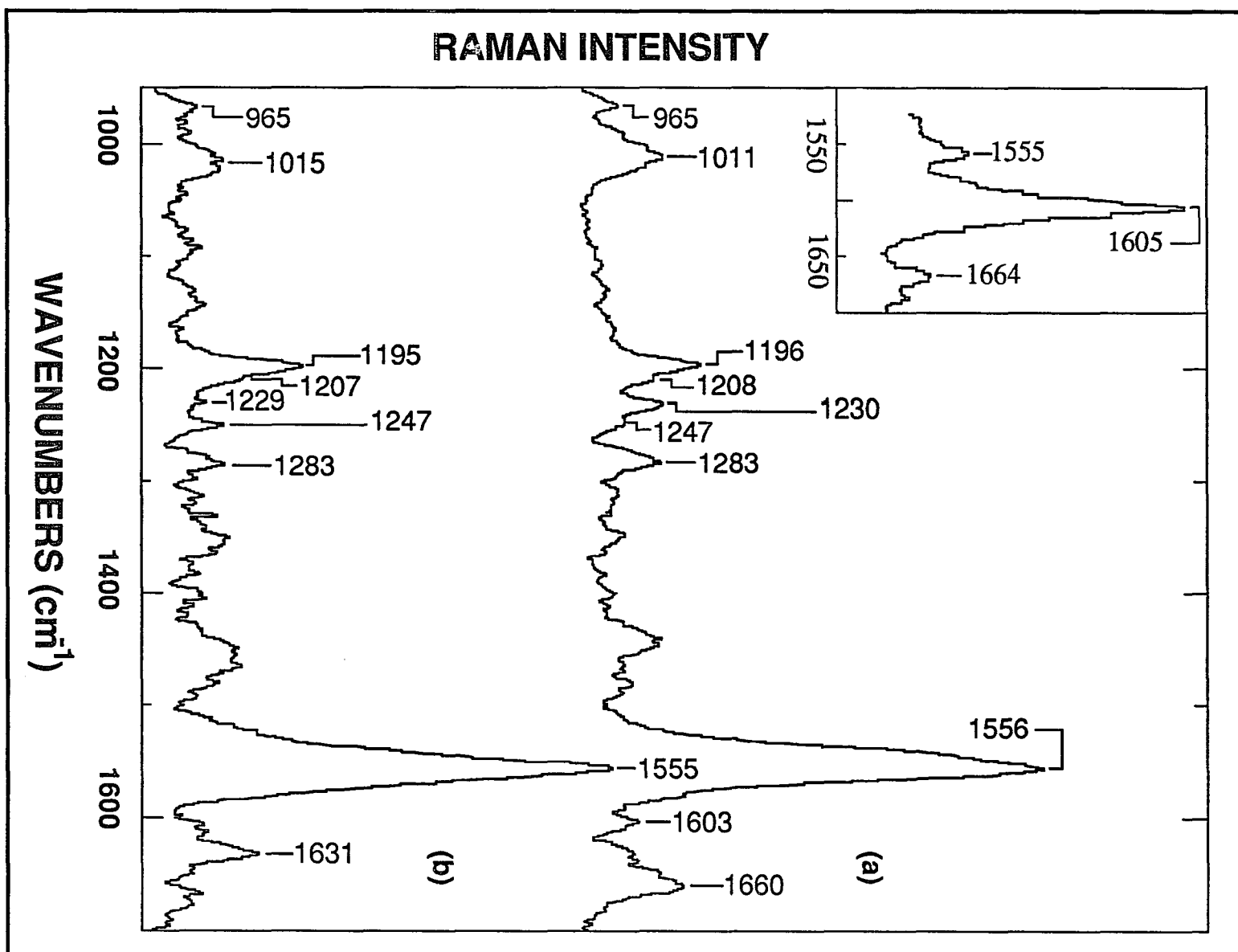


Figure 4.2

Figure 4.3 A plot of the absorption maximum vs. frequency of the most intense ethylenic band of some of the retinal pigment chromophores.

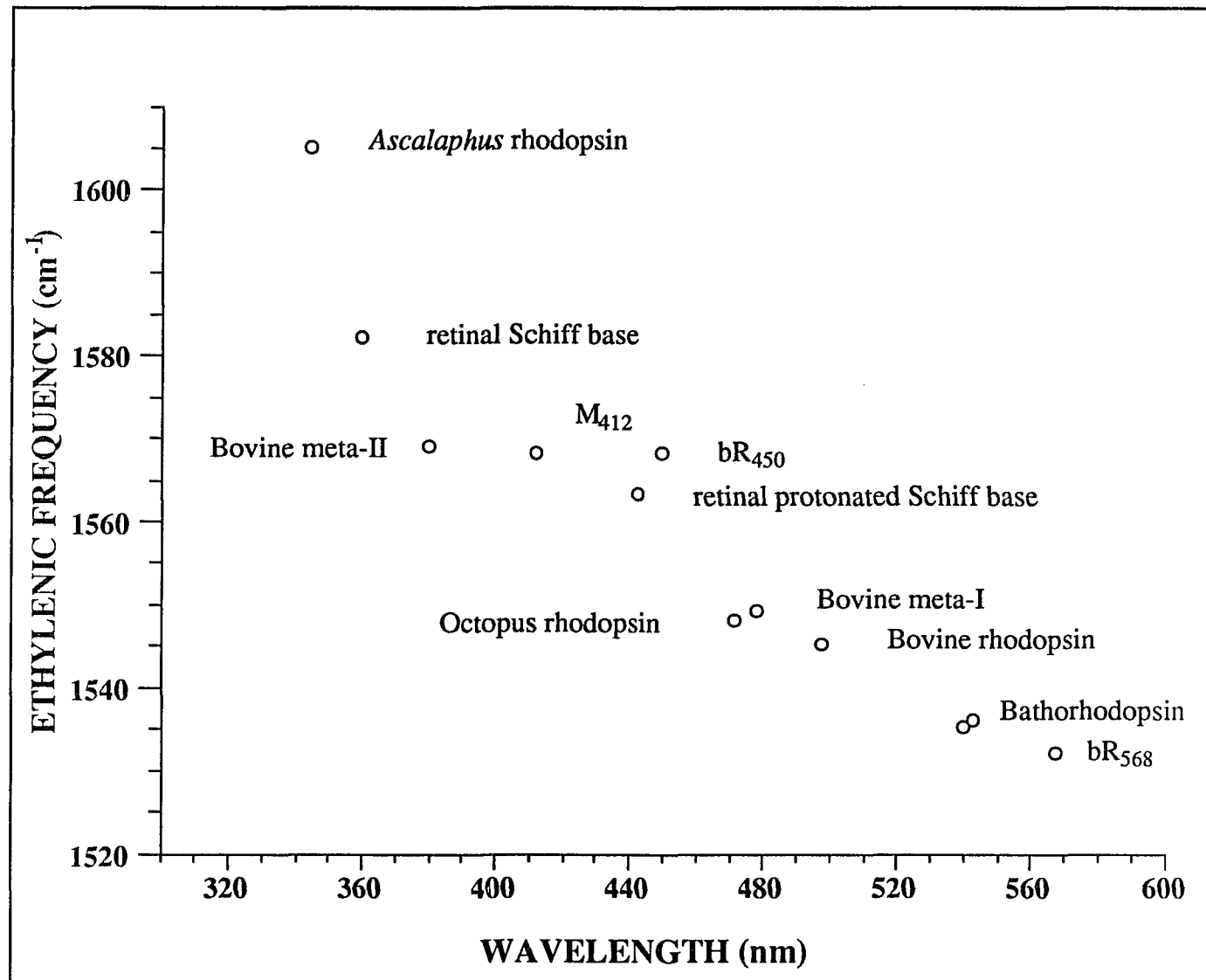


Figure 4.3

CHAPTER 5

Study of the HOOP modes in octopus visual pigment

5.1. Introduction

Upon absorbing a photon, octopus rhodopsin undergoes a series of photochemical and dark reactions (see chapter 1) which is observed to be similar to its bovine counterpart. Both bovine and octopus rhodopsins seem to share a common mechanism of storing incident photon energy, i.e. via isomerization (from 11-*cis* to 11-*trans* in their primary photoproduct) of their chromophores. All these similarities do not seem to be reflected in their Raman spectra which are quite different from each other (see e.g. Callender *et al.*, 1976; Pande *et al.*, 1987). There are interesting differences in the region below 1000 cm^{-1} where vinyl hydrogen out of plane (HOOP) vibrations are expected. Model chromophores do not show any strong intensities below 1000 cm^{-1} in their Raman spectra. Vibronic theory (Tang & Albrecht, 1970) predicts that the intensity of a resonance Raman transition depends upon the Franck-Condon factors (see chapter 1) which will be large for a particular vibration if the electronic excitation results in a displacement of the equilibrium position of that normal coordinate. Thus, displacement of the chain hydrogens out of the molecular plain would be expected to cause the intensity enhancement of the local HOOP modes. The QCFF- π calculations of Eyring *et al.* (1980) were able predict significant intensity enhancement of some of the HOOP modes by twisting the chromophore about 10-11 and 12-13 single bonds that results in the displacements from the molecular plain of some of the chain hydrogens. These displacements of the chain

hydrogens in a visual pigment chromophore could be obtained through specific protein-chromophore interactions. Rhodopsins of bovine and octopus show fewer HOOP modes compared to their respective bathorhodopsins. This might be a clue to the more strained chromophore structure in the bathorhodopsins. Although the chemical extraction data show that the octopus bathorhodopsin is predominantly all-*trans* (Pande *et al.*, 1987), its Raman spectrum is very different from that of the all-*trans* model compounds. This suggests that the protein interacts sufficiently with the chromophore to alter its vibrational behavior. There is a pronounced evidence of this in the HOOP region where the octopus bathorhodopsin has four strong lines at 885, 937, 954 and 967 cm^{-1} . In contrast, bovine bathorhodopsin has modes at 850, 858 (not very well resolved), 875 and 921 cm^{-1} (Palings *et al.*, 1989, Eyring *et al.*, 1982; also see figure 5.1). This suggests that the chromophore-protein interactions are not the same in octopus and bovine bathorhodopsins. Identification of the HOOP modes in bovine bathorhodopsin has provided clues to its structural and functional studies (Palings *et al.*, 1989). The purpose of our studies here is to resolve the observed differences between the bovine and octopus bathorhodopsin Raman spectra and investigate the chromophore structure of octopus bathorhodopsin. The first step towards achieving this goal is to identify all the HOOP modes and to do so we have undertaken studies on the octopus rhodopsins regenerated from deuterated chromophores. We have obtained low temperature Raman spectra of $\text{C}_{10}\text{-D}$ and $\text{C}_{12}\text{-D}$ 11-*cis* retinal regenerated octopus rhodopsin and are presented below.

5.2. Materials and methods

Octopus (*Paroctopus deflini*) microvillar membranes were prepared as described in Tsuda, 1986 and stored at -80°C until further use. Preparation of C₁₀-D and C₁₂-D 11-*cis* retinal analogues have been described in literatures (Pardoen *et al.*, 1986; Lugtenburg, 1985 and references cited therein) and was a generous gift from Prof. J. Lugtenburg of University of Leiden, Netherlands. Bleaching and regeneration of octopus rhodopsin with retinal analogues was carried out at University of Illinois, Urbana, according to the following procedure. Ten ml of membrane with a typical rhodopsin concentration of 0.2 OD at 570 nm was prepared in a 38% sucrose solution in 66mM Na/K phosphate buffer with 0.2M hydroxylamine (pH=6.5). This preparation was transferred to a glass culture tube in an ice-cold bath. Bleaching was carried out in dark room with a 400W slide projector using light of wavelengths longer than 480 nm (Corning cut off filter 3-70) and the sample was magnetically stirred throughout. Absorption spectra were obtained at regular intervals until complete bleaching was achieved, which takes typically about 60 min.

The bleached membranes were then washed four times (JA-20 rotor in a JA-21 Beckman centrifuge, for 30 min at 20k rpm) at 4°C with 66mM Na/K phosphate buffer (pH=7.0), to remove hydroxylamine from the preparation. After the final round of wash the pellet was suspended to the same final sample volume as during bleaching, in 38% sucrose solution in the same buffer used for washes. This was used for regeneration without further dilution.

About 100µl of 11-*cis* retinal (equivalent concentration of 2 OD/ml at 380 nm), either C₁₀-D or C₁₂-D analogue, in ethyl alcohol was added to the bleached membrane preparation described above and left at room

temperature for 1 $\frac{1}{2}$ hr. Regenerated rhodopsin thus obtained was added with 100 μ l of 2M hydroxylamine (pH 6.5), and washed two times in 5mM Tris buffer (pH 7), in the same way as in bleached membrane preparation. This was then washed twice more with 4% BSA solution in 5mM Tris buffer (pH=7). The final wash was carried out with just the Tris buffer as in the first two washes. All the washes were performed for 30 min at 20k rpm in a J2-21 Beckman centrifuge, JA-20 rotor. The pellet was suspended in 10 ml of 66 mM Na/K buffer (pH 7) with 38% (w/v) sucrose solution. The maximum regeneration yield obtained was 55%. Regenerated rhodopsin was stored at -20°C until Raman use.

For Raman experiments, a tight pellet of octopus rhodopsin was prepared by centrifuging 1 OD (at 475 nm in 1ml volume) of the regenerated rhodopsin for two hours at 13,000 rpm in a Sorval RC2-B centrifuge (SS-34 rotor). The pellet was transferred to a copper tip and mounted to the low temperature setup, described in section 2.3 of chapter 2, and was frozen quickly. The temperature of the sample was maintained at 90K throughout the experiment. The 457.9 nm beam from an argon ion laser (Spectra physics, Model 165) was used at the power level of ~3 mW for Raman excitation. Under these conditions the spectra represented mostly bathorhodopsin (~50%; Pande *et al.*, 1987) with some contributions from rhodopsin and isorhodopsin. Each of the sample spectrum was recorded for two hours.

5.3. Results

Figure 5.1 shows the comparison of bovine and octopus bathorhodopsins (courtesy of H. Deng, C. Pande and R.H. Callender). The broad band at 856 cm^{-1} is actually a combination of bands at 850 cm^{-1} and

858 cm^{-1} (Palings *et al.*, 1989). Spectra in figures 5.2, 5.3 and 5.4 are the low temperature (90 K) steady state Raman spectra of native and $\text{C}_{12}\text{-D}$ & $\text{C}_{10}\text{-D}$ regenerated octopus visual pigments respectively. As pointed out by Pande *et al.* (1987), the dominant contribution to these spectra comes from their respective bathorhodopsins. The native bathorhodopsin spectrum (figure 5.2) has four strong HOOP lines at 967, 954, 937 and 885 cm^{-1} . In the $\text{C}_{12}\text{-D}$ batho-rhodopsin spectrum (figure 5.3), a strong line is observed at 972 cm^{-1} , close to the 967 cm^{-1} line in the native spectrum. A relatively weaker band appears at 918 cm^{-1} and the 885 cm^{-1} band remains unchanged. No intensity is observed at 954 cm^{-1} and a new peak appears at 795 cm^{-1} . From these data the 954 cm^{-1} line can be assigned to an isolated $\text{C}_{12}\text{-H}$ HOOP which shifts to 795 cm^{-1} on deuteration. The H/D frequency ratio in this case is 1.2, amazingly close to the ratio for a totally isolated HOOP wag (Eyring *et al.*, 1982). Also two more new bands appear at 864 and 847 cm^{-1} in the 12- deuterated spectrum.

In the $\text{C}_{10}\text{-D}$ spectrum (Figure 5.4), all three lines at 967, 954 and 937 cm^{-1} are present in a redistributed intensity at the same frequencies as in native bathorhodopsin. Two new lines appear at 878 and 863 cm^{-1} . No strong intensity could be observed below these lines. The band at 885 cm^{-1} is absent. This suggests that the 885 cm^{-1} frequency is linked to $\text{C}_{10}\text{-H}$ mode but no conclusive answer can be obtained from these data.

5.4. Discussion

Resonance Raman spectrum of the primary photochemical intermediate in vision, bathorhodopsin, provides important clues to its chromophore structure. Comparison of the bovine and octopus bathorhodopsin Raman spectra could resolve their structural differences. A

primary step for such a comparative study is the identification of individual modes of vibration. The HOOP region of octopus bathorhodopsin contains four well separated strong modes at 885, 937, 954 and 967 cm^{-1} whereas bovine bathorhodopsin has strong modes at 850, 858, 875 and 921 cm^{-1} (Palings *et al.*, 1989). The three modes at 850, 875 and 921 cm^{-1} in the bovine bathorhodopsin has been assigned to isolated 14, 10 and 11 HOOP wags respectively by Mathies and coworkers (Eyring *et al.*, 1982; Palings *et al.*, 1989). They have also noted that the $\text{C}_{12}\text{-H}$ wag is strongly perturbed (probably due to a negatively charged protein residue near C_{12}) and may lie at 858 cm^{-1} (Palings *et al.*, 1989). Our $\text{C}_{12}\text{-D}$ substituted chromophore data shows that the $\text{C}_{12}\text{-H}$ wag of octopus bathorhodopsin has a much larger force constant than its bovine counterpart and the H/D frequency ratio in this case is 1.2. This suggests that the $\text{C}_{12}\text{-H}$ wag in this case is an isolated mode. This would also suggest that the 12-hydrogen of octopus bathorhodopsin is influenced by the protein very differently than in bovine bathorhodopsin and there may not be a charged protein residue near C_{12} . This must be verified with additional Raman data from other deuterated octopus samples. Although we tentatively assign the 885 cm^{-1} band to $\text{C}_{10}\text{-H}$ wag, no conclusive answer could be obtained from the data presented here. Further studies on other substituted octopus rhodopsins will help resolve the differences observed in the Raman spectra of octopus and bovine visual pigments.

Figure 5.1 Comparison of the resonance Raman spectra of (a) bovine bathorhodopsin and (b) octopus bathorhodopsin. Spectrum (b) is actually a photostationary mixture due to the 457.9-nm probe beam at 88 K. The bovine bathorhodopsin spectrum was obtained by appropriate subtractions of the photostationary spectra obtained with the 488-nm probe beam, in the absence and presence of appropriate pump beams, at 80 K. Spectral resolution was 8 cm⁻¹. (Courtesy of H. Deng, C. Pande and R.H. Callender)

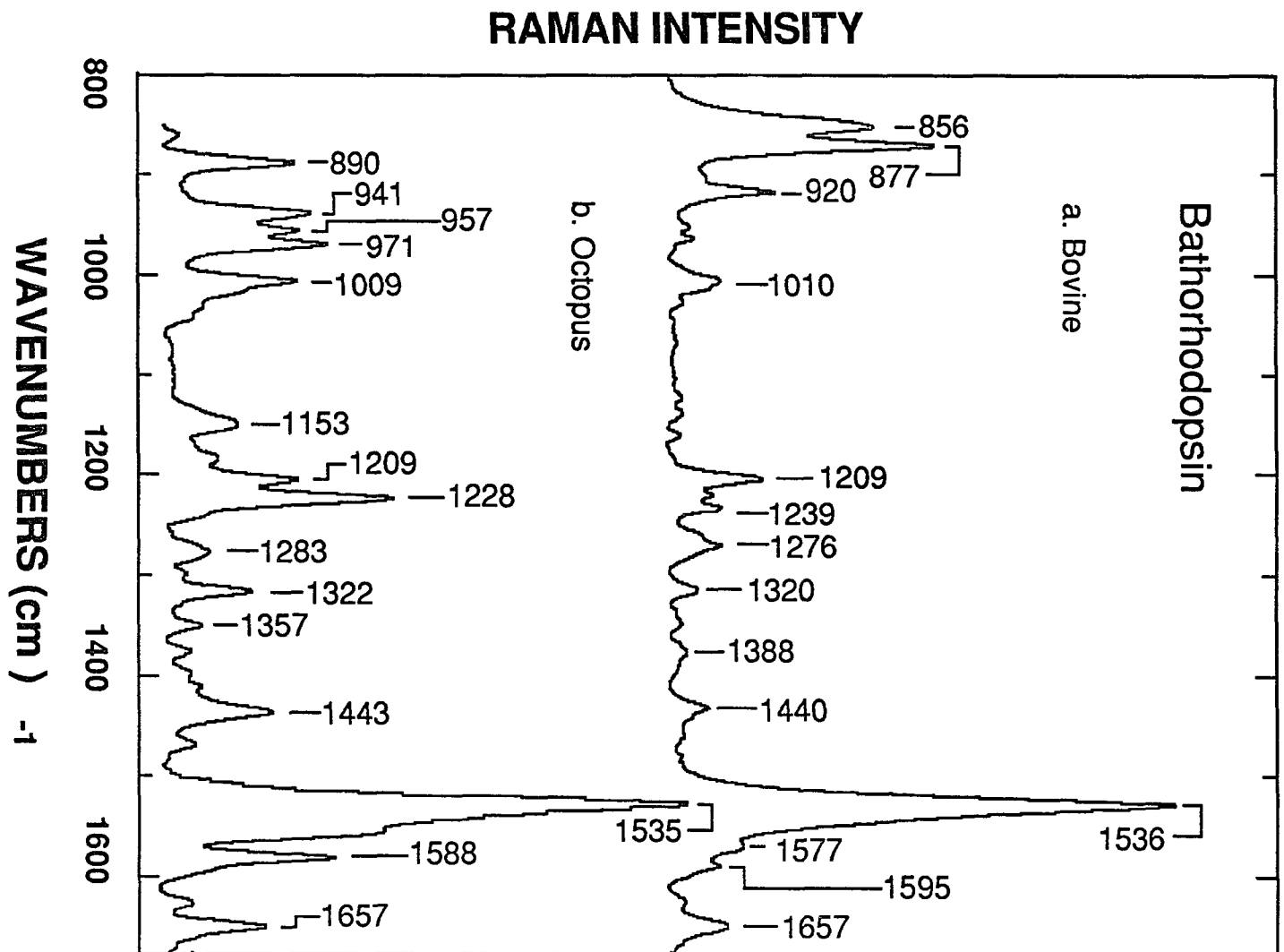


Figure 5.1

Figure 5.2 90K Raman spectrum of native octopus visual pigment.
The 457.9 line of an argon ion laser was used at the power level of 3mW for Raman excitation. Spectral resolution is 7 cm^{-1} and the peak positions are accurate up to 3 cm^{-1} .

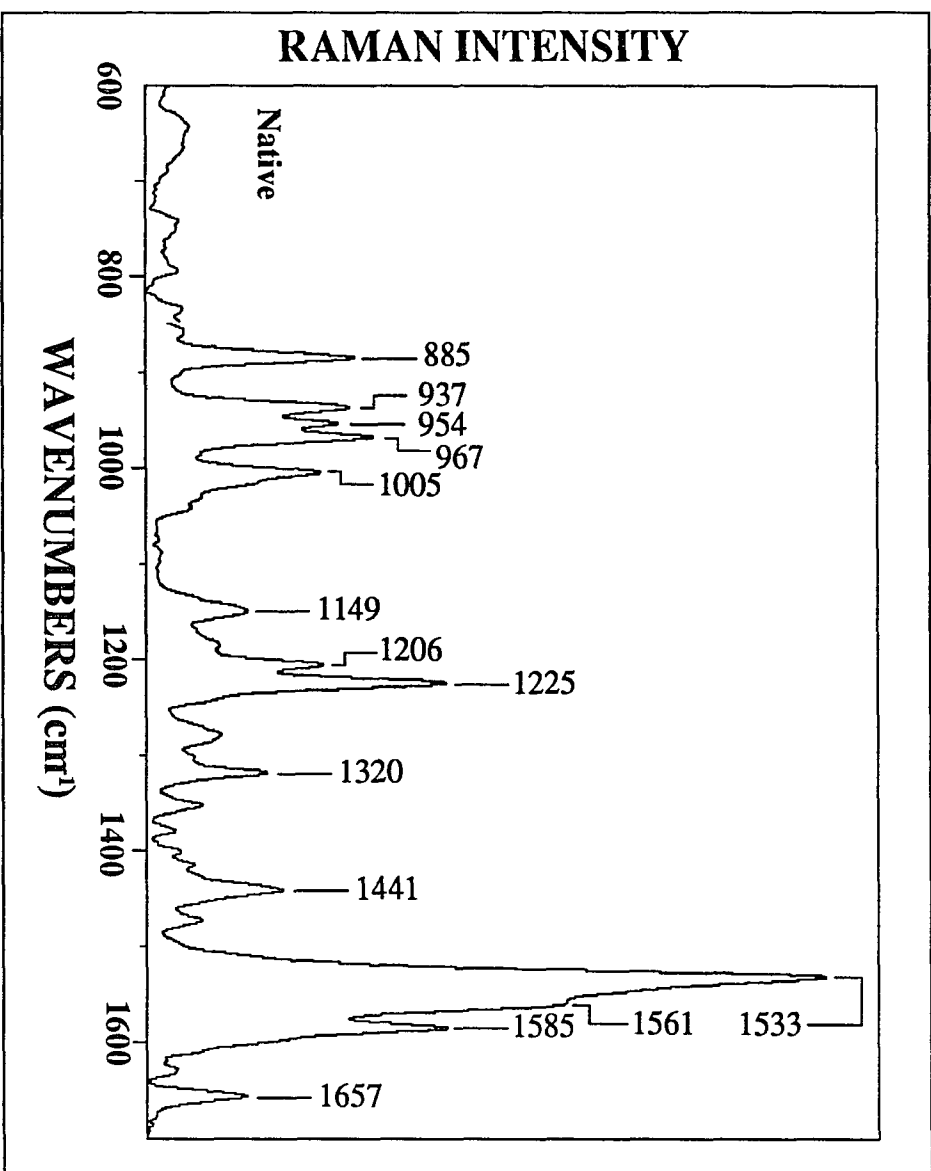


Figure 5.2

Figure 5.3 90K Raman spectrum of C₁₂-D all-*trans* retinal regenerated octopus visual pigment. The 457.9 line of an argon ion laser was used at the power level of 3mW for Raman excitation. Spectral resolution is 7 cm⁻¹ and the peak positions are accurate up to 3 cm⁻¹.

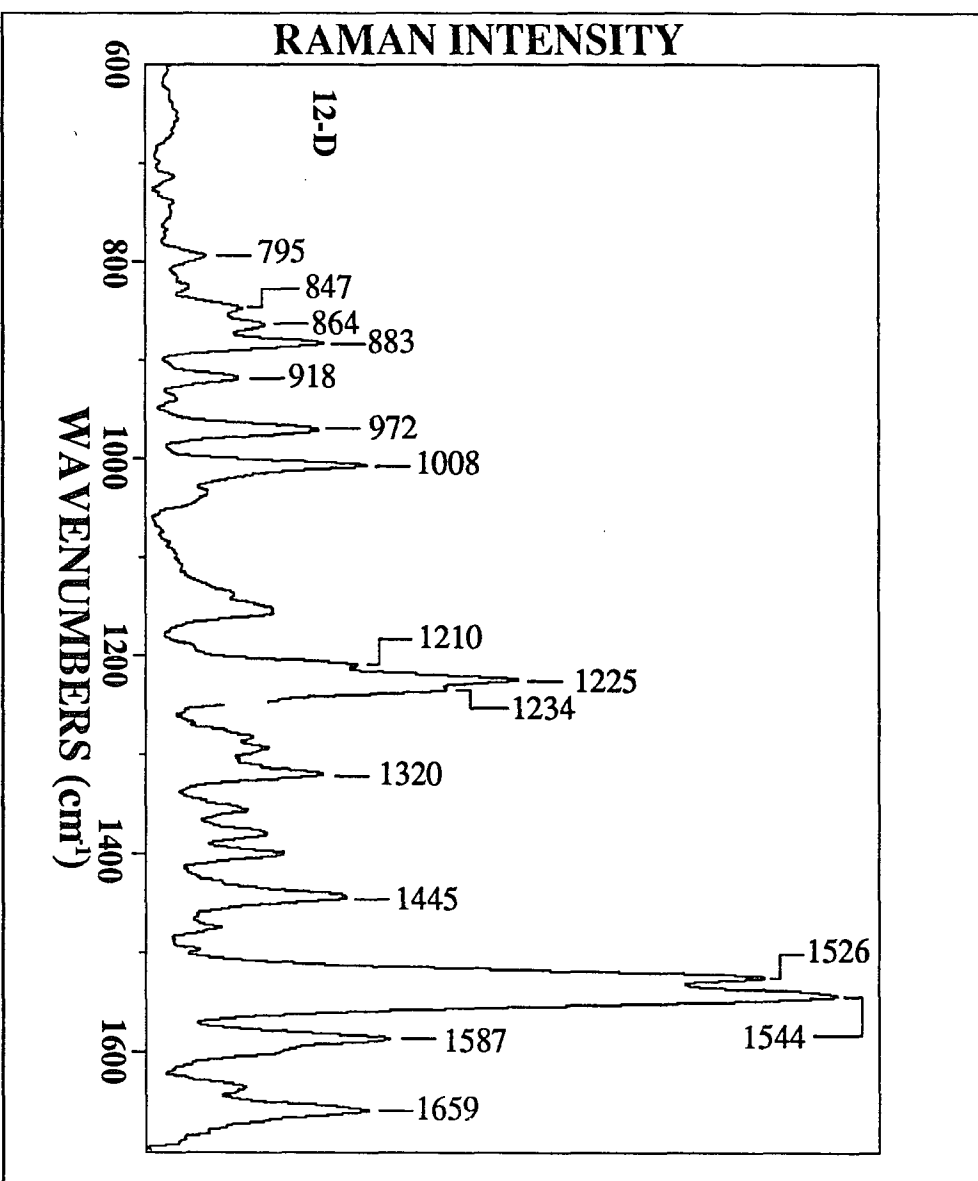


Figure 5.3

Figure 5.4 90K Raman spectrum of C₁₀-D all-*trans* retinal regenerated octopus visual pigment. The 457.9 line of an argon ion laser was used at the power level of 3mW for Raman excitation. Spectral resolution is 7 cm⁻¹ and the peak positions are accurate up to 3 cm⁻¹.

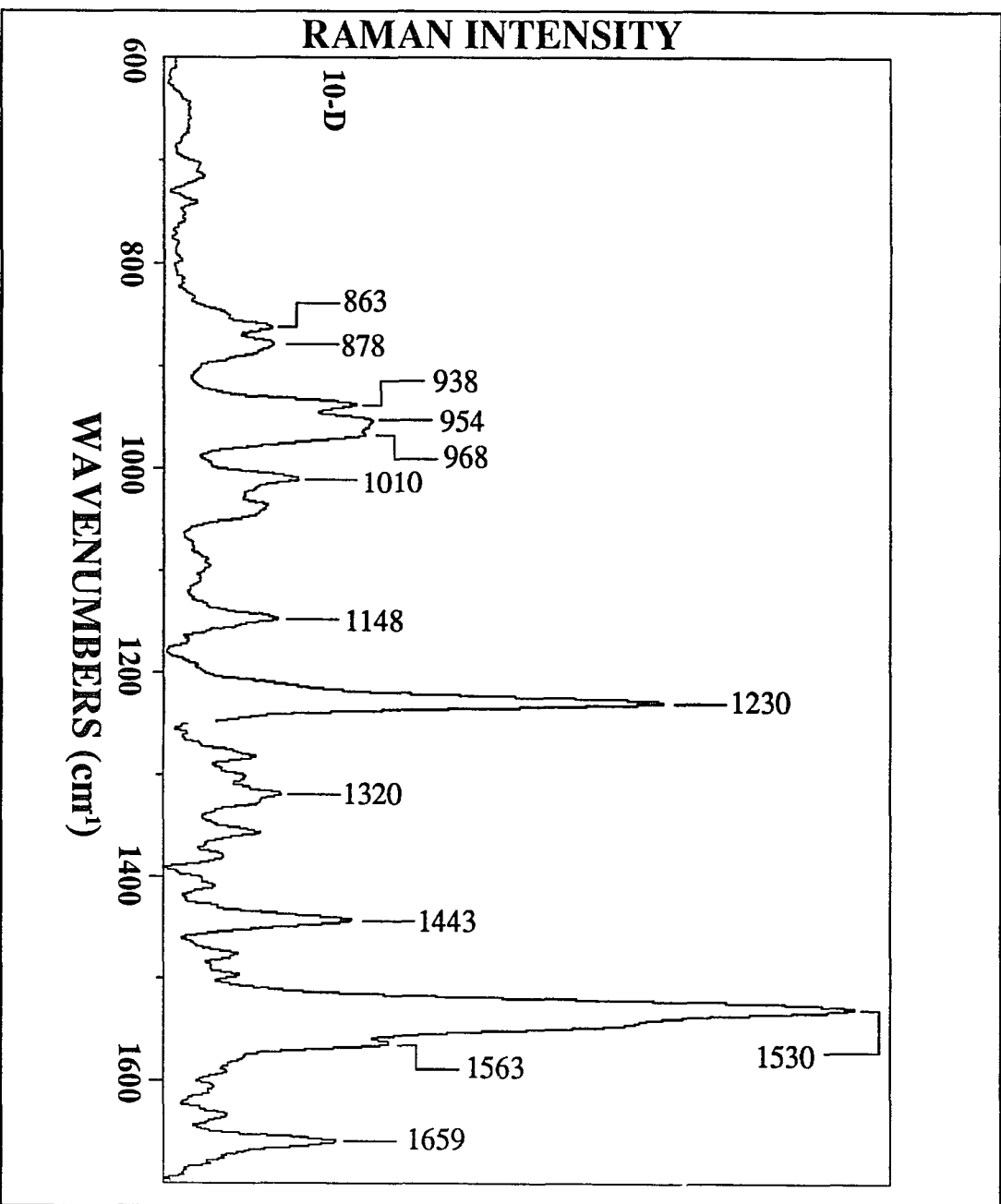


Figure 5.4

CHAPTER 6

A comparative study of Bacteriorhodopsin at neutral and alkaline pH

6.1. Introduction

The trans-membrane purple protein bR is synthesized by the extreme halophilic *Halobacterium halobium* under low oxygen supply condition. This protein stores light energy in the form of an electrochemical proton gradient across the cell membrane by pumping protons out of the cell (see chapter 1). Upon absorption of light, light-adapted bR undergoes a photochemical cycle consisting of several distinct intermediate species which are linked to a vectorial proton translocation across the cell membrane. A linear reaction scheme of these intermediates, K₆₁₀, L₅₅₀, M₄₁₂, N, O₆₄₀ etc. (figure 1.7), was first proposed by Lozier *et al.* (Lozier *et al.*, 1975; Lozier & Niederberger, 1977) on the basis of optical transient (flash photolysis) and low temperature difference spectroscopy. This simple scheme was later found to be unsuitable to accommodate the multi exponential decay kinetics of some of the intermediates (see e.g. Groma and Dancshäzy, 1986). Various modifications to this linear scheme were suggested that include a sequential reaction pathway (Lozier *et al.*, 1978), a parallel branched pathway (see e.g. Li *et al.*, 1984; Groma *et al.*, 1984) and a single species in equilibrium with other intermediates (Parodi *et al.*, 1984). Some of the recent works in this field (Hanamoto *et al.*, 1984; Diller and Stockburger, 1988; Dancshäzy *et al.*, 1988) suggest, either directly or indirectly, that the multi exponential decay kinetics observed for some of the photo-intermediates are due to simultaneous decay of different

intermediate species originating from different forms of bR in equilibrium with each other. According to the scheme of Dancshäzy *et al.* (1988) there could possibly be three forms of bR equilibrating with each other in a pH dependent kinetics, and each intermediate is associated with the photocycle of a particular form of bR (figure 6.1). Also, Stockburger and coworkers (Diller and Stockburger, 1988) have proposed a scheme in which two forms of bR with distinct individual photo-intermediates equilibrate with each other.

Our goal here is to look for a Raman spectroscopic evidence of these proposed schemes. Since pH is often cited as an important parameter in these schemes (Dancshäzy *et al.*, 1988; Diller and Stockburger, 1988) we have obtained the Raman spectra of bR at neutral pH and under high pH (pH 10) with high salt concentration, and the results are presented below.

6.2. Materials and methods

Bacteriorhodopsin was a generous gift from Prof. Thomas G. Ebrey of the University of Illinois, Urbana and was stored at 4°C until further use. Raman spectra were obtained from the sample flowing through the laser beam at a speed of 700 cm/sec inside a glass capillary of 0.5 mm internal diameter. A peristaltic pump was used to flow this sample from a 100 ml transparent home made conical glass reservoir. The outer jacket of this reservoir was cooled by water/ethylene glycol mixture from a Lauda circulator bath. The flow setup is described in more detail in chapter 2. Sixty ml of bR in 1.5M NaCl, 15 mM piperazine glycylglycine buffer adjusted to desired pH (6.5 or 10), was used for each experiment and the temperature of the sample was maintained at 10°C. Typically, the absorbance of bR was ~3.8 OD at 570 nm. Light adaptation of bR was

carried out in the flow setup using light from a 250W slide projector with a orange glass filter (corning 3-70) for several minutes until no increase in absorbance at 586 nm (wavelength of maximum differential absorption between light and dark-adapted bR) was observed. Sufficient time (3 min for pH 6.5 sample and 5 min for pH 10 sample) was allowed following light adaptation for the photo-intermediates to relax before accumulating Raman spectra so as to avoid spectral contamination. The sample was magnetically stirred throughout the duration of the experiment. Raman signal was accumulated for 2-3 min in each step consisting of no more than 3 transits of sample through the probe beam and about 3-5 min time was allowed between successive Raman excitations to allow the probe generated intermediates (if any) to decay. Raman signal was summed for one hour of total accumulation. None of the Raman data presented in this chapter were averaged. The spectra thus recorded had a resolution of 6 cm⁻¹.

Experimental arrangement was made such that a sample molecule flowing through the probe beam had a very low probability of absorbing a photon. This is essential since any contribution from a photo-intermediate in resonance with the probe beam will lead to misleading results in the difference Raman spectra of ground state bR. A cylindrically focussed 514.5 nm laser beam from an argon ion laser was used for the Raman excitation at a power level of 1 mW near the sample. The total number of photons absorbed and causing isomerization through a single transit of the sample molecule is given by the relation

$$P_m = (\text{laser flux})(\text{absorption cross section})(\text{quantum yield})(\text{time of transit through the beam})$$

where P_m is the photoalteration parameter. In the case of a cylindrical lens the focus lies in a plane. If this focal plane has a width w in the direction of the sample flow and a dimension d perpendicular to it, the laser flux will be $I_o/(wd)$ where I_o is the intensity of the laser beam (photons/sec). Now, the photoalteration parameter can be symbolically written as

$$\begin{aligned} P_m &= [I_o/(wd)](\sigma_a)(\psi)(w/v_b) \\ &= [I_o/(v_b d)](\sigma_a \psi) \end{aligned}$$

where σ_a is the absorption cross section, ψ is the quantum yield, v_b is the speed of sample flow. The absorption cross section σ_a is given by $3.824 \times 10^{-21} \epsilon$ where ϵ is the extinction coefficient. Using 1 mW of 514.5 nm wavelength as the probe beam and assuming the following values for the parameters,

$$\sigma_a = 3.824 \times 10^{-21} \epsilon$$

$$\epsilon = 35000 \text{ M}^{-1} \text{ cm}^{-1}$$

$$\psi = 0.3 \text{ for bR (Becher and Ebrey, 1977)}$$

$$d = 0.5 \text{ mm}$$

$$v_b = 700 \text{ cm/sec}$$

we obtain the value of $P_m = 0.003$. In this limit the incident photon has less than 1% chance of being absorbed by the sample molecule and initiate photocycle in it during a single pass. This ensures that no appreciable contamination of the Raman signal is caused by the probe generated photo-intermediates.

The spectrum of bR with a long lived photo-intermediate (N) was obtained in a way similar to the pH 10 bR experiment described above with some modifications. The spectra were recorded continuously from a

flowing sample while the sample reservoir being flooded with light from a 250W slide projector equipped with corning 3-70 orange colored glass filter. The sample (bR) was in a 1.5M NaCl and 15mM piperazine glycylglycine buffer at pH 10 and was magnetically stirred throughout. Because of the continuous illumination with orange light the long lived bR intermediate(s) is(are) expected to be accumulated in the sample over a period of time. The transit time from the reservoir to the probing beam was ~1 sec and is long enough for all the short lived photo-intermediates to decay before being detected by the probing beam. The K₆₁₀ (λ_{max} = 610 nm), M₄₁₂ (λ_{max} = 412 nm) and O₆₄₀ (λ_{max} = 640 nm) intermediates have very little chances of being detected by the probe beam (514.5 nm) because of nonresonant condition. Although the L₅₅₀ (λ_{max} = 550 nm) intermediate will be in resonance with the probe (514.5 nm), it is expected to be depleted within the 1 sec transit time from the reservoir to the probe beam. The spectra thus recorded had contributions mainly from light-adapted bR and long lived photo-intermediate(s).

6.3. Results

6.3a. bR at pH 6.5 & pH 10

Figures 6.2 (a) and (b) show the Raman spectra of light adapted bR at pH 10 and pH 6.5 respectively. All the major peaks of the pH 6.5 spectrum seem to be present in the pH 10 spectrum. The most intense ethylenic band ($\nu_{\text{C}=\text{C}}$) of both the samples appear at the same frequency (within the experimental error) but the FWHM of pH 10 spectrum is ca. 5 cm^{-1} more compared to that at pH 6.5. A wider FWHM could arise when two or more species are present in the sample. Additional intensities are also observed at ~1187 and 1175 cm^{-1} in the pH 10 spectrum. These

changes can be observed more clearly when pH 6.5 spectrum is subtracted (72%) from that of pH 10, as shown in figure 6.3(a). Additional peaks are resolved at 1175 and 1545 cm^{-1} . The main ethylenic band appears at 1529 cm^{-1} with a clear shoulder at 1545 cm^{-1} . All of these observations suggest that two or more species might be contributing to the pH 10 spectrum. From the inverse correlation between λ_{max} and C=C stretching frequency ($\nu_{\text{C}=\text{C}}$) (Aton *et al.*, 1977, Doukas *et al.*, 1978; also see e.g. figure 4.3), a $\nu_{\text{C}=\text{C}}$ value of 1529 cm^{-1} will correspond to a λ_{max} of ~560 nm and the 1545 cm^{-1} ethylenic stretch will correspond to a still lower value of λ_{max} . This clearly suggests that the alkaline (pH 10) spectrum has contributions from some additional species absorbing at lower wavelengths than bR^{LA} (λ_{max} =568nm). The shoulder on the low frequency side of the main ethylenic band in figure 6.3(a) shows that there is some contribution from the species having $\nu_{\text{C}=\text{C}}$ at 1526 cm^{-1} (bR^{LA}) to this spectrum. Further subtraction (92%) of pH 6.5 spectrum from that of pH 10 completely removes this contribution and is shown in figure 6.3(b). Two ethylenic bands at 1531 and 1547 cm^{-1} with unequal intensities are observed in figure 6.3(b). This spectrum resembles very much like that of the N photo-intermediate (see the text below) recently reported (Fodor *et al.*, 1988).

6.3b. The N intermediate

Figure 6.4(a) is the light adapted bR spectrum at pH 6.5 [same as figure 6.2(b)] and figure 6.4(b) is the spectrum at pH 10 of light-adapted bR under illumination (see materials and methods). The peak at 1187 cm^{-1} appearing in this spectrum [figure 6.4(b)] is also observed in the spectrum of light-adapted bR at pH 10 [figure 6.2(a)]. In addition to this, there is a clear shoulder on the higher frequency side of the main ethylenic band.

Figure 6.4(c) is obtained when the spectrum shown in figure 6.4(a) (pH 6.5, bR^{LA}) is subtracted from the spectrum of figure 6.4(b) (light-adapted bR under illumination at pH 10). This spectrum [figure 6.4(c)] is expected to have contributions mostly from a long lived photo-intermediate (see materials and methods) and is very similar to the N spectrum reported by Mathies and coworkers (Fodor *et al.*, 1988). For example, in their spectrum of the N intermediate the Schiff base mode appears at 1642 cm⁻¹ and the ethylenic band consists of a doublet with unequal intensities at 1530 and 1548 cm⁻¹. In our difference spectrum [figure 6.4(c)] the Schiff base mode also appears at 1642 cm⁻¹ and the ethylenic band is a doublet with unequal intensities at 1531 and 1547 cm⁻¹. The peaks at 1186, 1198 and 1210 cm⁻¹ reported in their N spectrum are also present at or around the same frequencies in our spectrum of the long lived intermediate. These similarities suggest that our spectrum of pH 10 light-adapted bR under illuminated condition has contribution from the long lived N intermediate of the bR photocycle.

It is also seen that figure 6.4(c) (N intermediate) is very much similar to figure 6.3(b) (difference spectrum between pH 6.5 and pH 10). All the major peaks seen in figure 6.3(b), e.g. peaks at 1583, 1547, 1531, 1257, 1203, 1187 and 1178 cm⁻¹, are also present in the spectrum of the long lived photo-intermediate N [figure 6.4(c)] at the same frequencies. This shows that the difference observed between pH 6.5 and pH 10 bR could be due to the presence of some amount of the N intermediate in the pH 10 sample. This is not surprising since Kouyama *et al.* (1988) have noted that at room temperature the relaxation time of the N intermediate to bR could be as high as 10 sec, hence the presence of some amount of N photo-intermediate in our experiment can be expected.

6.4. Discussion

Recent transient absorption studies (Dancshäzy *et al.*, 1986; Drachev *et al.*, 1986; Kouyama *et al.*, 1988) have identified conditions under which a long lived intermediate (R, P or N) of the bR photocycle can be studied. All of these studies have shown that under alkaline pH condition a new photo-intermediate, R, P or N can be observed which is not seen under physiological conditions. Stockburger and coworker (Alsuth and Stockburger, 1986) had also observed a new photo-intermediate in alkaline bR (pH=9.6), they named it as "L", in their kinetic resonance Raman experiments. A consensus seem to emerge among all the workers regarding the presence of a new photo-intermediate at alkaline pH which is not observed at neutral pH. Mathies and coworkers (Fodor *et al.*, 1988) have reported the Raman spectrum of the N intermediate and have placed it between M₄₁₂ and O₆₄₀ intermediates of the bR photocycle. Our results also show the presence of a long lived bR photo-intermediate at pH 10 and high ionic strength (1.5M NaCl) condition and the spectrum is very similar to the N spectrum reported in the literature.

The complex rise and decay kinetics of the R, P, N intermediates have led to the suggestion of a possible different form of bR (Dancshäzy *et al.*, 1988; Kouyama *et al.*, 1988). Diller and Stockburger (1988) have ascribed the inhomogeneous kinetic behavior of the L₅₅₀ intermediate to two different conformational states of the apoprotein giving rise to two different states of bR. El-Sayed and coworkers (Hanamoto *et al.*, 1984) had suggested that the biphasic kinetics seen in the rise of M₄₁₂ are due to two chromophore environments provided by the apoprotein. In essence all of the above workers have suggested the existence of at least two forms of

bR existing in a pH dependent equilibrium. Except for the presence of some amount of a long lived photoproduct (i.e. the N intermediate) in the pH 10 spectrum, we did not observe any difference in the Raman spectra of bR at pH 6.5 and pH 10. This however, does not prove or disprove the suggestion of the existence of two or more forms of bR but it can be said that the two forms of bR might have very similar Raman spectra which are difficult to resolve.

Figure 6.1 Sketch of the various pH dependent photocycles of bacteriorhodopsin as proposed by Dancshazy *et al.* (1988). The two forms of L and M intermediates have been associated with two forms of bR with different λ_{max} , and the R intermediate (Dancshazy *et al.*, 1986) has been associated with a third form of bacteriorhodopsin.

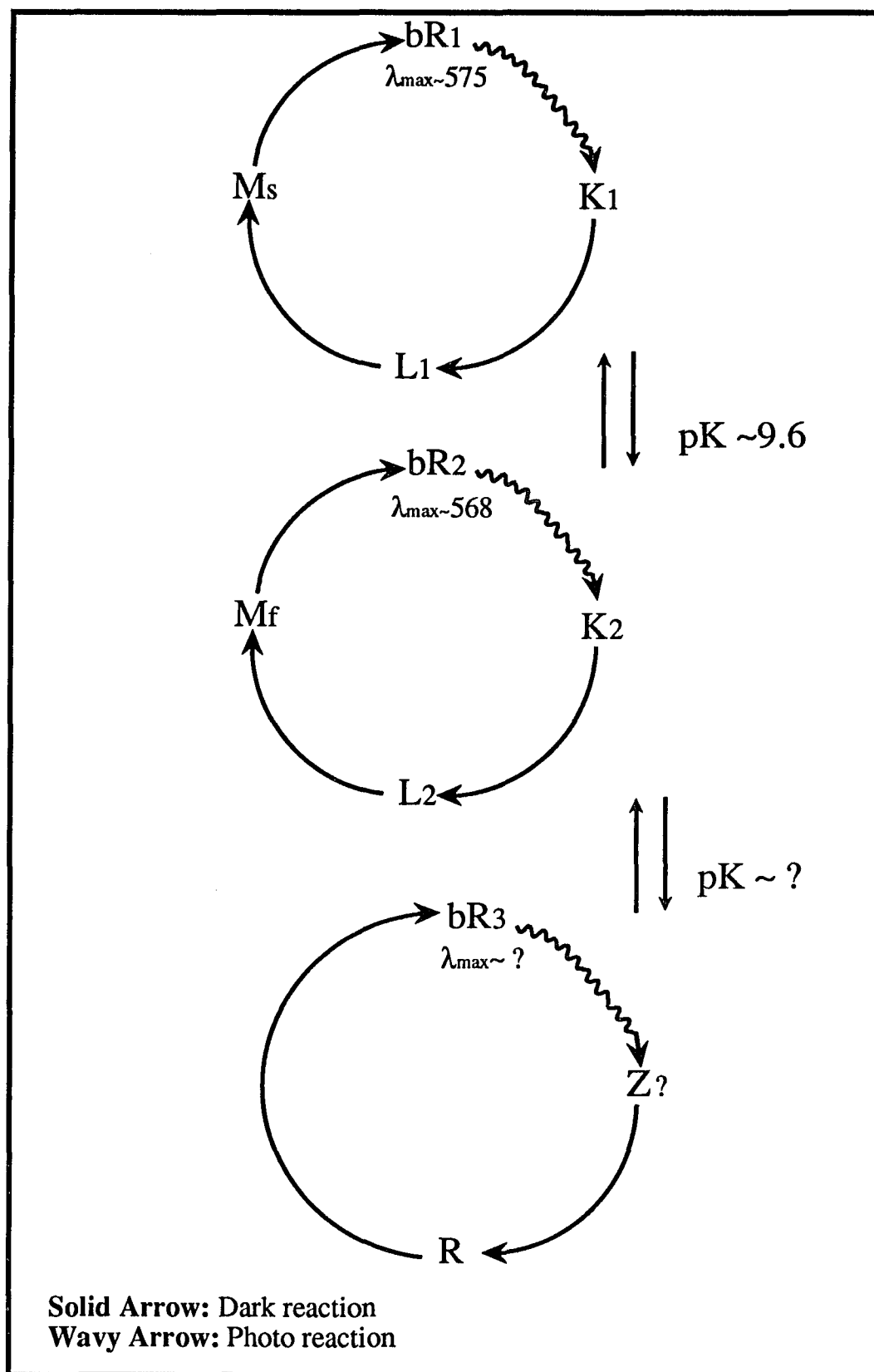


Figure 6.1

Figure 6.2 Flow resonance Raman spectrum of light-adapted bR at pH 6.5 (a) and pH 10 (b). Samples were in 15mM piperazine glycilglycine buffer and 1.5M NaCl. Flow speed was 700 cm/sec and the temperature was $\sim 10^{\circ}\text{C}$. The 514.5 nm line of an argon ion laser was used at the power level of 1mW for Raman excitation. Spectra represent the response of OMA after dark current subtraction, no averaging was performed.

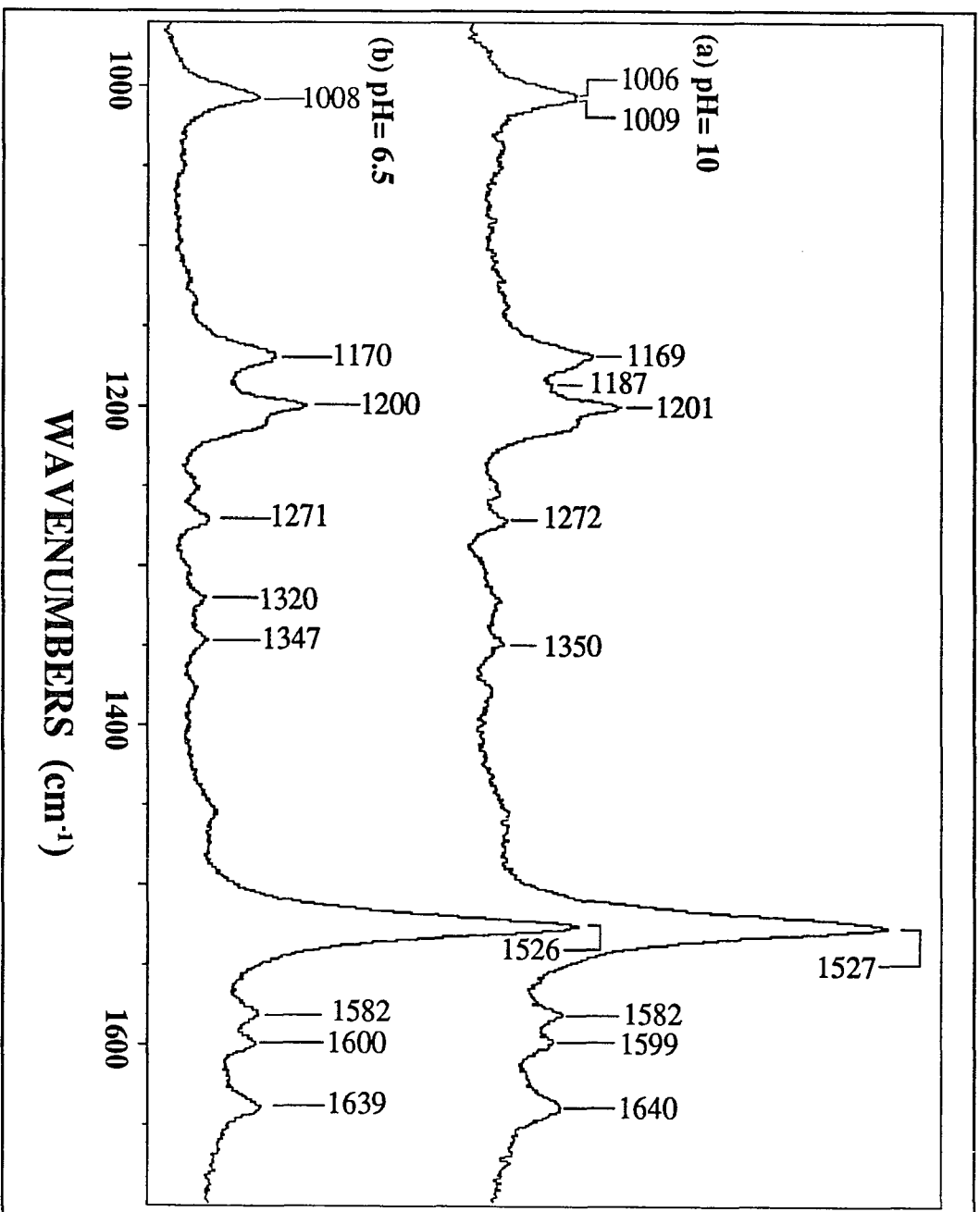


Figure 6.2

Figure 6.3 Raman difference spectra of bR between pH 6.5 and pH 10. Figure (a) was obtained after subtracting 72% of pH 6.5 bR [figure 6.2(b)] from the pH 10 spectrum of light-adapted bR [figure 6.2(a)]. Figure (b) was obtained after 92% subtraction of pH 6.5 spectrum from that of the pH 10. Spectra were normalized to the same highest counts before subtractions.

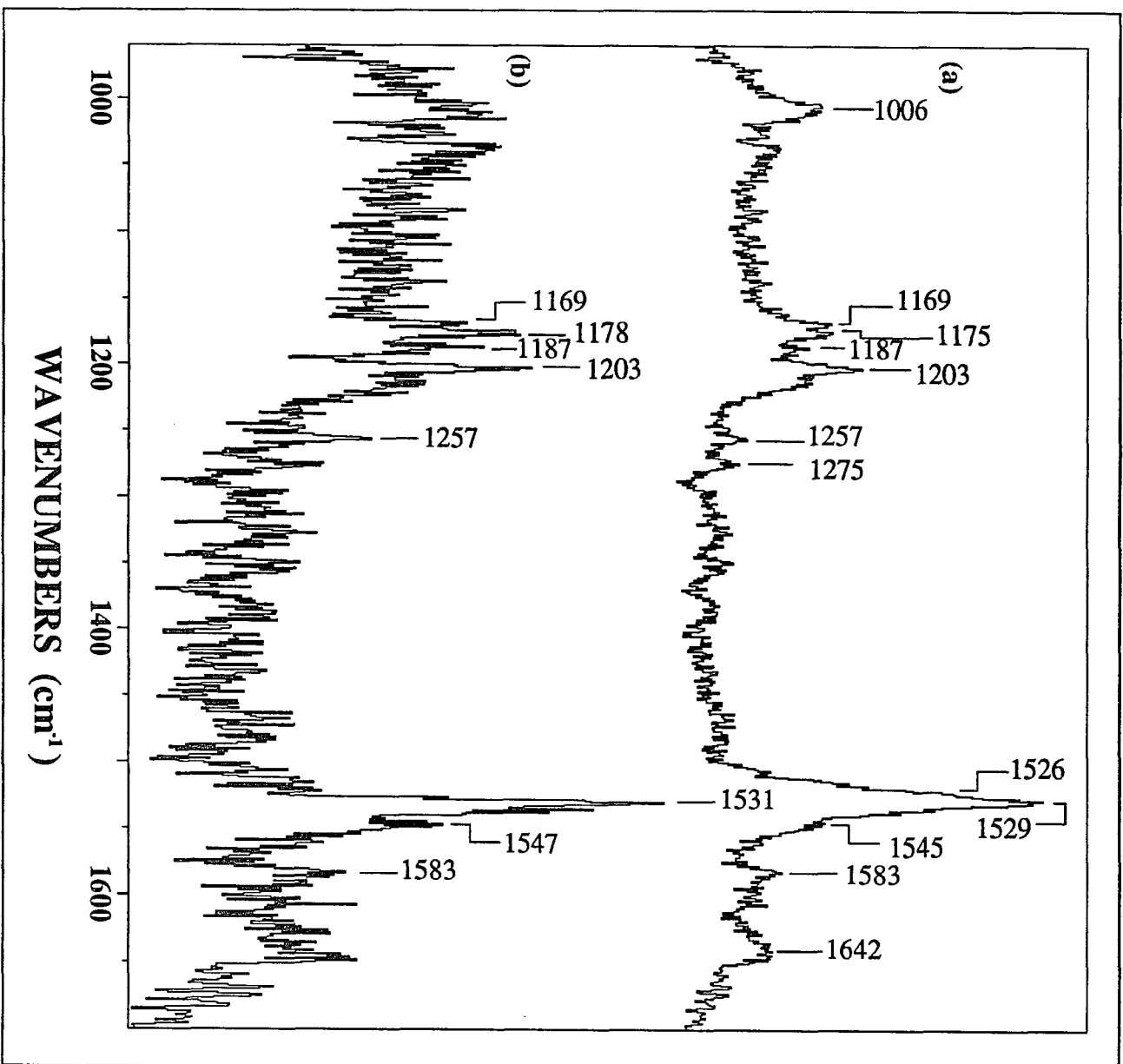


Figure 6.3

Figure 6.4 Flow resonance Raman spectrum of bR at pH 6.5 (a) and pH 10 under illumination (b). Figure (a) is same as figure 6.2(b). Spectrum (b) was recorded from a flowing bR sample (flow speed 700cm/sec) while the sample reservoir was illuminated with 250W orange light. Figure (c) was obtained after subtracting maximum possible amount of spectrum (a) from that of (b) such that no negative peaks appear in the subtracted spectrum.

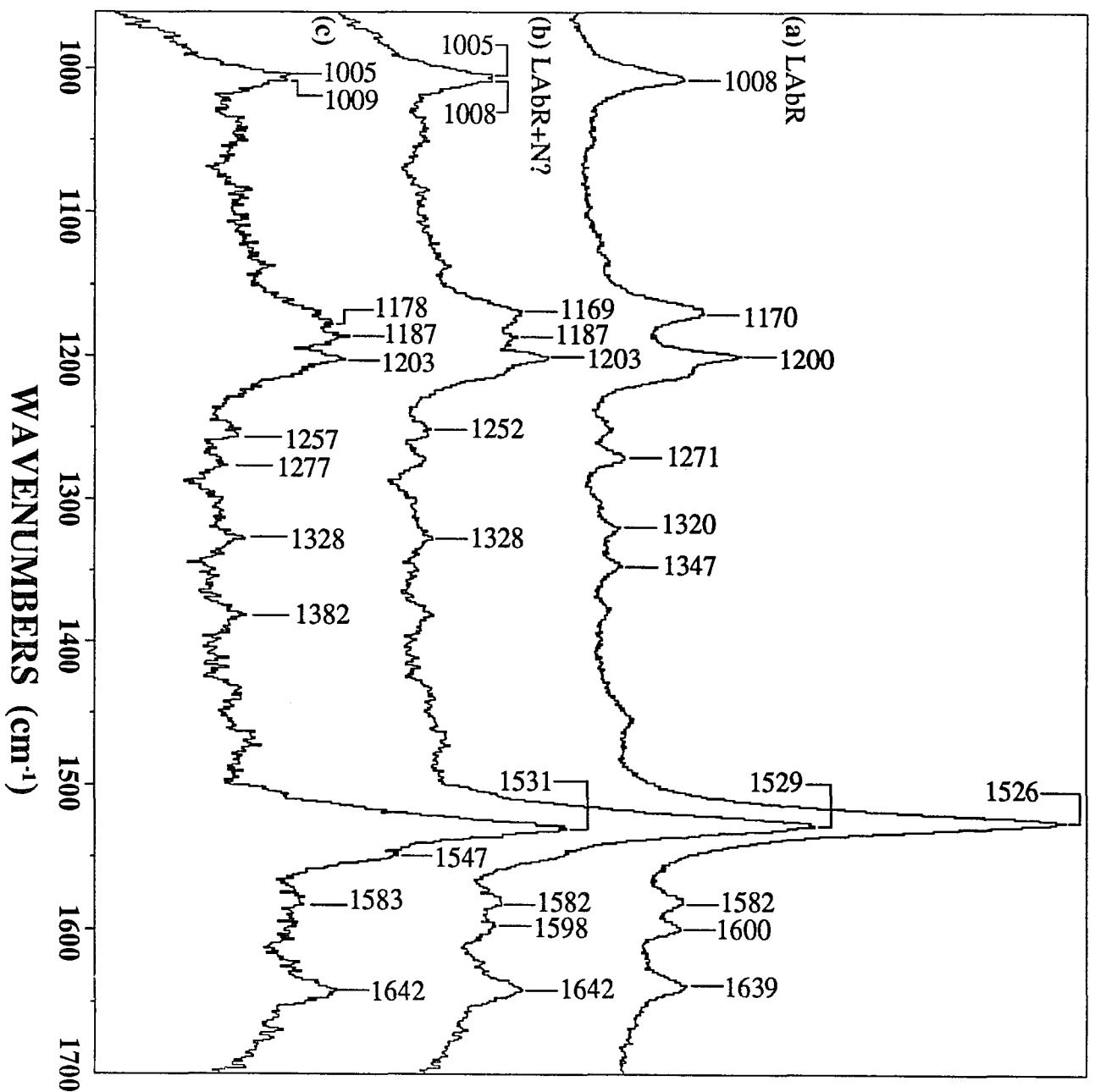


Figure 6.4

CHAPTER 7

Study of a Bacteriorhodopsin analogue:

13-bromo-bR

7.1. Introduction

The purple protein bacteriorhodopsin (bR) of the microorganism *Halobacterium halobium* acts as a light driven proton pump. The electrochemical energy generated by pumping protons across the cell membrane is utilized for cell metabolism. There are two forms of bR: the light-adapted form (bR^{LA}, $\lambda_{\max}$ = 568 nm) has an all-*trans* chromophore and in the dark, bR^{LA} converts to the dark-adapted form (bR^{DA}, $\lambda_{\max}$ ~ 560 nm) containing a mixture of all-*trans* and 13-*cis* chromophores. It has been observed that upon absorption of a photon, the light adapted form of bR (bR^{LA}) undergoes a photocycle through several intermediate species of various colors as shown in figure 1.7 of chapter 1 (Lozier *et al.*, 1975; Lozier & Niederberger, 1977). The first step in this photocycle is the isomerization of the protonated all-*trans* chromophore of bR^{LA} to the 13-*cis* form in the first intermediate K₆₁₀ of the photocycle. At least one deprotonated intermediate species, M₄₁₂ (Aton *et al.*, 1977; Braiman and Mathies, 1980; Tsuda *et al.*, 1980a) has been observed in this cycle and this intermediate is believed to play a key role in the proton pumping mechanism. A detailed description of the bR photocycle and properties of its intermediates are given in chapter 1.

Regeneration of bR is possible from bR-opsin and all-*trans* retinal (see chapter 3) and the regenerated pigment has $\lambda_{\max}$ at ~570 nm. Bacteriorhodopsin has also been regenerated from bR-opsin and all-*trans*

13-bromo retinal analogue ($=C_{13}-CH_3-$ of the retinal replaced by $=C_{13}-Br-$, see figure 7.1) (Motto *et al.*, 1980). In this case a blue colored pigment is formed having the λ_{max} at 595 nm (figure 7.1). If left in dark for several hours at room temperature, a little or no dark adaptation of this pigment was found suggesting that thermal isomerization of its chromophore (all-*trans* to 13-*cis*) is seriously hindered. Since proton pumping is linked to the photocycle and the initial step in the photocycle is the isomerization of the chromophore, proton pumping will not be possible if isomerization is blocked. Contrary to this hypothesis, the proton pumping efficiency of 13-bromo-bR was found to be of the same order as for native bR (B.N. Rao and K. Nakanishi; unpublished data). This raised the question whether the photo-isomerization of 13-bromo-bR is possible. We have studied this isomerization behavior and the nature of the proton pumping mechanism in 13-bromo-bR and the results are presented here.

7.2. Materials and methods

The 13-bromo-bR was prepared by adding all-*trans* 13-bromo retinal (figure 7.1) to the bleached membrane of bacteriorhodopsin and was a generous gift from Professor K. Nakanishi's group at Columbia University. In order to remove the unreacted retinal, 0.2M hydroxylamine was added and the sample was washed with 4% BSA (w/v) three times by centrifugation. The pigment thus obtained had an absorption maximum at 595 nm. Absorption measurements were carried out in a Perkin-Elmer spectrophotometer described in section 2.1 of chapter 2. For the Raman measurements, ~1 ml of the sample having a typical optical density of 1 at 595 nm was taken in a 5 cm diameter circular plexyglass spinning cell described in chapter 2 (section 2.5). The cell was spun at a speed of 2700

rpm by an electrical motor. The sample was light adapted using a 200W slide projector and a orange filter (Corning 3-70) for 20 min. No appreciable change in the absorption was observed, suggesting that the sample was already in the light adapted form. A cylindrically focussed 568.2 nm beam from a krypton ion laser (Coherent, Model CR-2000) was used for obtaining the spectra of the light adapted form of the pigment. Two cylindrically focussed coaxial laser beams were used to obtain the spectrum of the M photo-intermediate: the 568.2 nm line of a krypton ion laser was used to initiate the bR photocycle and the 457.9 nm beam of an argon ion laser was used downstream from it as the probe beam. The typical pump to probe power ratio was 5:1. When the separation between pump and probe beam was ~ 0.2 cm, the time separation between them was ~ 0.3 millisecond. Under these experimental conditions, the M_{412} intermediate of native bR is expected to be detected by the probe beam (Deng *et al.*, 1985). The spectrum of 13-bromo-bR with contributions from L intermediate was obtained from the sample spinning at a speed of 2700 rpm using ~ 100 mW of 514.5 laser beam which is expected to be in resonance. The beam diameter in this case was $\sim 80 \mu\text{m}$. Under these conditions and assuming a value of $35000 \text{ M}^{-1}\text{cm}^{-1}$ for the extinction coefficient (ϵ) of bR at 514 nm, we obtain the photoalteration parameter P_m to be ~ 2.08 (Callender *et al.*, 1976). This ensures the photo-excitation of every bR molecule passing through the beam. The transit time of a molecule through the beam is $\sim 11 \mu\text{sec}$, long enough for a significant accumulation of L intermediate in the beam (for comparison, the rise time of L_{550} is $\sim 1 \mu\text{sec}$). Thus under the above conditions the L intermediate is expected to be simultaneously generated and detected by the probe beam. All the measurements were carried out at room temperature. A simple two-point

averaging of the data was performed for all the Raman spectra reported in this chapter.

7.3. Results and discussion

7.3a. Light-adapted 13-bromo-bR

Shown in figure 7.2 is the Raman spectrum of light-adapted 13-bromo-bR in the spinning cell at 568.2 excitation. The transit time of a sample molecule through the probe beam was 11 μ sec and its return path was 22 msec. Under these experimental conditions, the Raman spectrum of native light-adapted bR (bR^{LA}) is not expected to have major contributions from its photo-intermediates because of the non-resonant condition and/or short life times of the intermediates. However, if the bR species we are looking at has a slower kinetics, its O intermediate could contribute to the Raman signal because of a pre-resonance condition and the millisecond decay time of this intermediate (e.g. O₆₄₀ of normal bR decays in ~5msec). In such a situation, following the empirical inverse correlation between $\nu_{C=C}$ vs. λ_{max} (Aton *et al.*, 1977; Doukas *et al.*, 1978; also see e.g. figure 4.3), the ethylenic band is expected to be broad and/or with distinct shoulders indicating the presence of multiple species. In our spectrum of 13-bromo-bR (λ_{max} = 595 nm), the main ethylenic band ($\nu_{C=C}$ stretch) appears at 1510 cm⁻¹ with a narrow FWHM value (17 cm⁻¹) and no distinct shoulder is observed. This suggests that we are looking at only one species which is the light-adapted form of 13-bromo-bR. Some contributions from the K intermediate can not be completely ruled out. The Schiff base frequency appears at 1645 cm⁻¹ which is close to its value for native light-adapted bR (1640 cm⁻¹). This shows that the presence of bromine atom at

C₁₃ does not cause any drastic change of protein-chromophore interactions in the Schiff base region. Comparison of figure 7.2 (13-bromo-bR) with figure 6.2b (bR^{LA}) shows that the structure sensitive fingerprint region (1100-1400 cm⁻¹) of 13-bromo-bR is very much similar to that of the native light-adapted bR (bR^{LA}). The peaks at 1347, 1322, 1198 and shoulder at 1210 cm⁻¹ in the 13-bromo-bR spectrum also appears in the bR^{LA} spectrum either at or around the same frequencies. Since native bR^{LA} has been shown to contain all-*trans* chromophore (Lewis *et al.*, 1974; Aton *et al.*, 1977; Pettei *et al.*, 1977; Tsuda *et al.*, 1980a; Braiman and Mathies, 1980), the similarities in the fingerprint region suggests that the chromophore in the 13-bromo-bR is also all-*trans*. Besides the main ethylenic band, the two additional C=C stretching modes at 1582 and 1600 cm⁻¹ in native bR^{LA} spectrum are also present in 13-bromo-bR spectrum at 1576 and 1598 cm⁻¹. The methyl stretch in 13-bromo-bR appears at 1016 cm⁻¹ instead of at 1008 cm⁻¹ in bR^{LA}. This is the expected frequency of the C₉-CH₃ mode when uncoupled by the presence of bromine atom at C₁₃ (Curry *et al.*, 1982). The HOOP region of 13-bromo-bR contains at least four modes at 812, 843, 868 and 950 cm⁻¹.

7.3b. The M intermediate

Figure 7.3 is the dual beam pump-probe spectrum of 13-bromo-bR. Under these conditions the M intermediate (M₄₁₂, λ_{max} = 412 nm) of native bR^{LA} is expected to be the dominant species contributing to the Raman spectrum (see section 7.2; Deng *et al.*, 1985). In the spectrum of figure 7.3, the main ethylenic band appears at 1558 cm⁻¹ instead of 1568 cm⁻¹ for M₄₁₂ (Deng *et al.*, 1985). A 1558 cm⁻¹ ethylenic stretch will correspond to a λ_{max} of ~440 nm from the empirical ν_{c=c} and λ_{max} correlation (Aton *et*

al., 1977, Doukas *et al.*, 1978; figure 4.3), e.g. the $\nu_{c=c}$ of all-*trans* PSB in solution ($\lambda_{\max} \sim 440$ nm) appears at ~ 1557 cm^{-1} (Aton *et al.*, 1977; Pande *et al.*, 1981). The only photo-intermediate which absorbs around this wavelength is M_{412} . This suggests that the 1558 cm^{-1} band corresponds to the M of 13-bromo-bR and the $\lambda_{\max}$ of this M intermediate is more than that of M_{412} (from the $\lambda_{\max}$ and $\nu_{c=c}$ correlation, figure 4.3, see discussion below). The Schiff base frequency of the M photo-intermediate of 13-bromo-bR appears at 1613 cm^{-1} . Such a low value for a Schiff base mode is expected from an unprotonated species. For example the Schiff base mode of the unprotonated intermediate M_{412} , appears at 1622 cm^{-1} whereas the protonated Schiff base modes of bR^{LA} , L_{550} , N_{550} , bR^{DA} appear at ~ 1640 cm^{-1} (Deng *et al.*, 1985; Smith *et al.*, 1985; Fodor *et al.*, 1988). The structure sensitive fingerprint region of our spectrum is very much similar to the spectrum of M_{412} (Deng *et al.*, 1985). Two strong bands at 1181 and 1196 cm^{-1} in our spectrum have their counterparts at 1183 and 1197 cm^{-1} in M_{412} . The methyl stretch in both M_{412} and M of 13-bromo-bR appears at 1013 cm^{-1} . The 977 cm^{-1} mode of M_{412} is seen at 974 cm^{-1} in our spectrum. All of these results suggest that the spectrum presented here is that of the unprotonated M intermediate of 13-bromo-bR and this M intermediate has similar isomeric conformation (13-*cis*) as that of M_{412} . An ethylenic frequency at ~ 1538 cm^{-1} is also seen in our spectrum of the M intermediate. Ethylenic mode around this frequency is observed only for the L_{550} intermediate of normal bR (Smith *et al.*, 1984; Alsuth and Stockburger, 1986). Since the L intermediate is not expected to be in resonance with the probe beam (457.9 nm), the 1538 cm^{-1} band in our spectrum of the M intermediate is not likely to be from the L of 13-bromo-bR.

7.3c. The L intermediate

The spectrum in figure 7.4 is very similar to that of light-adapted 13-bromo-bR (figure 7.2) with some differences. The main ethylenic band at 1509 cm^{-1} suggests that the spectrum contains contributions mostly from that of light-adapted 13-bromo-bR ($\lambda_{\text{max}} = 595\text{ nm}$) (see section 7.3.a) but the shoulder at 1525 cm^{-1} is probably due to some contributions from a species with λ_{max} around 580 nm (from λ_{max} vs $\nu_{\text{C}=\text{C}}$ correlation, Aton *et al.*, 1977, Doukas *et al.*, 1978). Comparison of λ_{max} of 13-bromo-bR (595 nm) with the λ_{max} of that of the intermediates in the native bR ($\lambda_{\text{max}} = 568\text{ nm}$) photocycle suggests that the most likely species to have absorption at $\sim 580\text{ nm}$ is the L intermediate. The higher intensity at 1172 cm^{-1} in this spectrum (figure 7.4) is probably due to the presence of the L intermediate.

7.4. Conclusions

The resonance Raman data presented here strongly favors an all-*trans* protonated Schiff base chromophore for the light-adapted form of 13-bromo-bR. Like native bR (bR^{LA}), the 13-bromo-bR undergoes photocycle upon irradiation and an unprotonated intermediate (M) is observed. This is consistent with the observations of Rao *et al.* (B.N. Rao and K. Nakanishi, unpublished results) that the 13-bromo-bR pumps protons efficiently. The nature and isomeric configuration of this M intermediate is similar to that of M₄₁₂. The difference in energy between bR^{LA} ($\lambda_{\text{max}} = 568\text{ nm}$) and its M₄₁₂ intermediate ($\lambda_{\text{max}} = 412\text{ nm}$) is $\sim 6670\text{ cm}^{-1}$. When this difference is incorporated in 13-bromo-bR ($\lambda_{\text{max}} = 595\text{ nm}$) the M intermediate is expected to have $\lambda_{\text{max}} \sim 426\text{ nm}$. However, the empirical λ_{max} vs. $\nu_{\text{C}=\text{C}}$ correlation predicts a λ_{max} of $\sim 440\text{ nm}$ for the M intermediate of 13-bromo-bR. These observations suggest that the λ_{max} of the M intermediate of 13-

bromo-bR is most probably higher than that of M_{412} and may have a value around 430 nm. Similarly, incorporation of the $\sim 580\text{ cm}^{-1}$ energy difference between bR^{LA} and L_{550} intermediate in to the 13-bromo-bR predicts a λ_{max} of $\sim 575\text{ nm}$ for its L intermediate. Although the data presented here are not sufficient to identify the L intermediate of 13-bromo-bR, it suggests that this intermediate could have a λ_{max} around 580 nm on the basis of $\nu_{C=C}$ and λ_{max} correlation. Our observations strongly suggest that the chromophore of 13-bromo-bR is quite efficient in photoisomerization although there is evidence of its thermal isomerization being hindered (see section 7.1). Its photocycle is very similar to that of the native bR and hence they must have the common mechanism for pumping protons.

Figure 7.1 (a) Sketch of an all-*trans* 13-bromo retinal. (b) Spectral absorbance of 13-bromo-bR showing the pigment absorption maximum at 595 nm.

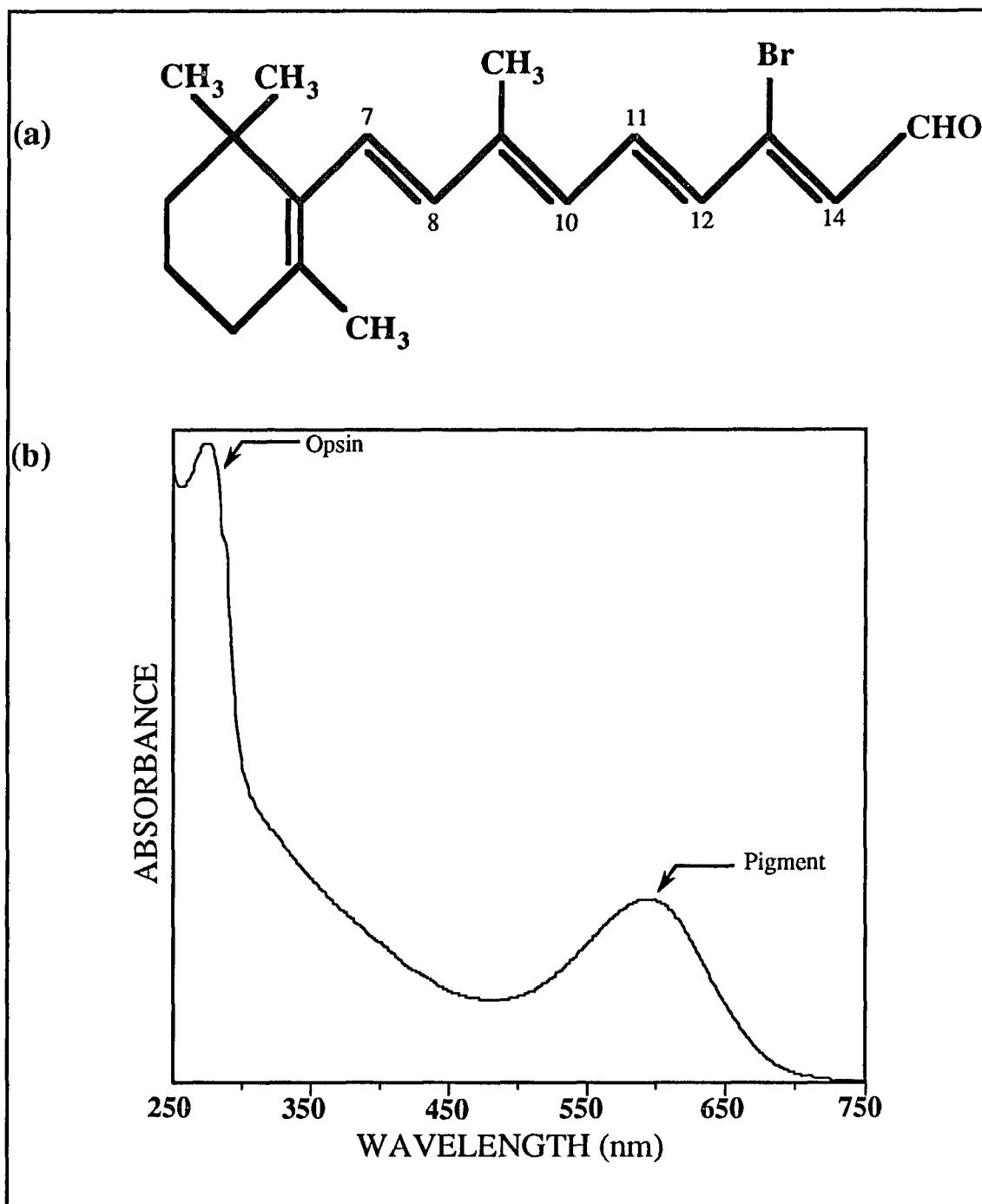


Figure 7.1

Figure 7.2 Resonance Raman spectrum of light-adapted 13-bromo-bR from a rotating spinning cell at room temperature. The 568.2 nm beam of a krypton ion laser was used for Raman excitation. Spectra were recorded in two windows (700-1250 cm^{-1} and 1250-1700 cm^{-1}) and joined together. The Spectral resolution was $\sim 6 \text{ cm}^{-1}$.

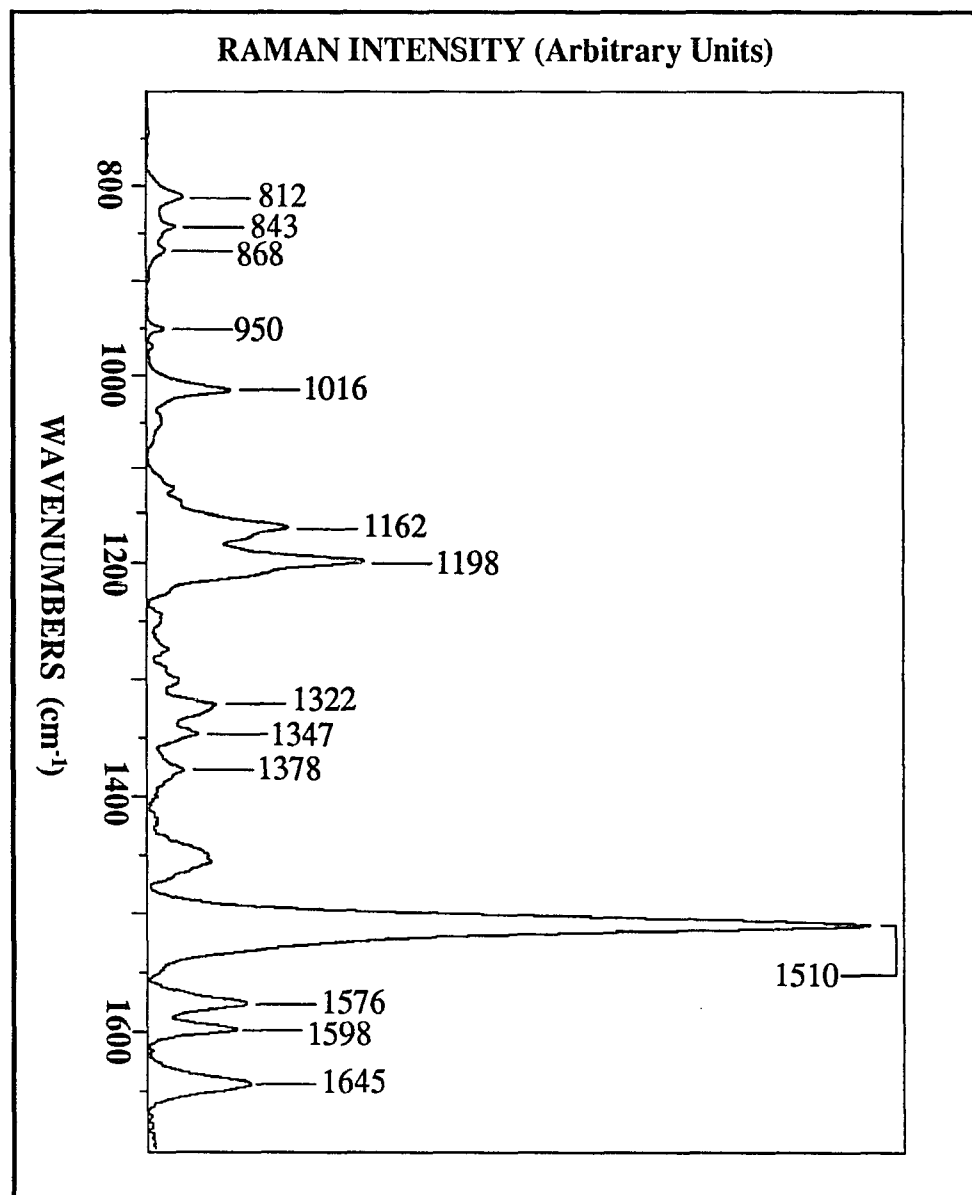


Figure 7.2

Figure 7.3 The dual beam pump-probe Raman spectrum of light-adapted 13-bromo-bR representing its M intermediate. The sample was in a rotating spinning cell at room temperature. The 457.9 nm line of an argon ion laser was used as the probe beam and the 568.2 nm line of a krypton ion laser was used to initiate the photocycle. Pump to power ratio was 5:1 and the time separation between them was ~0.3 millisecond.

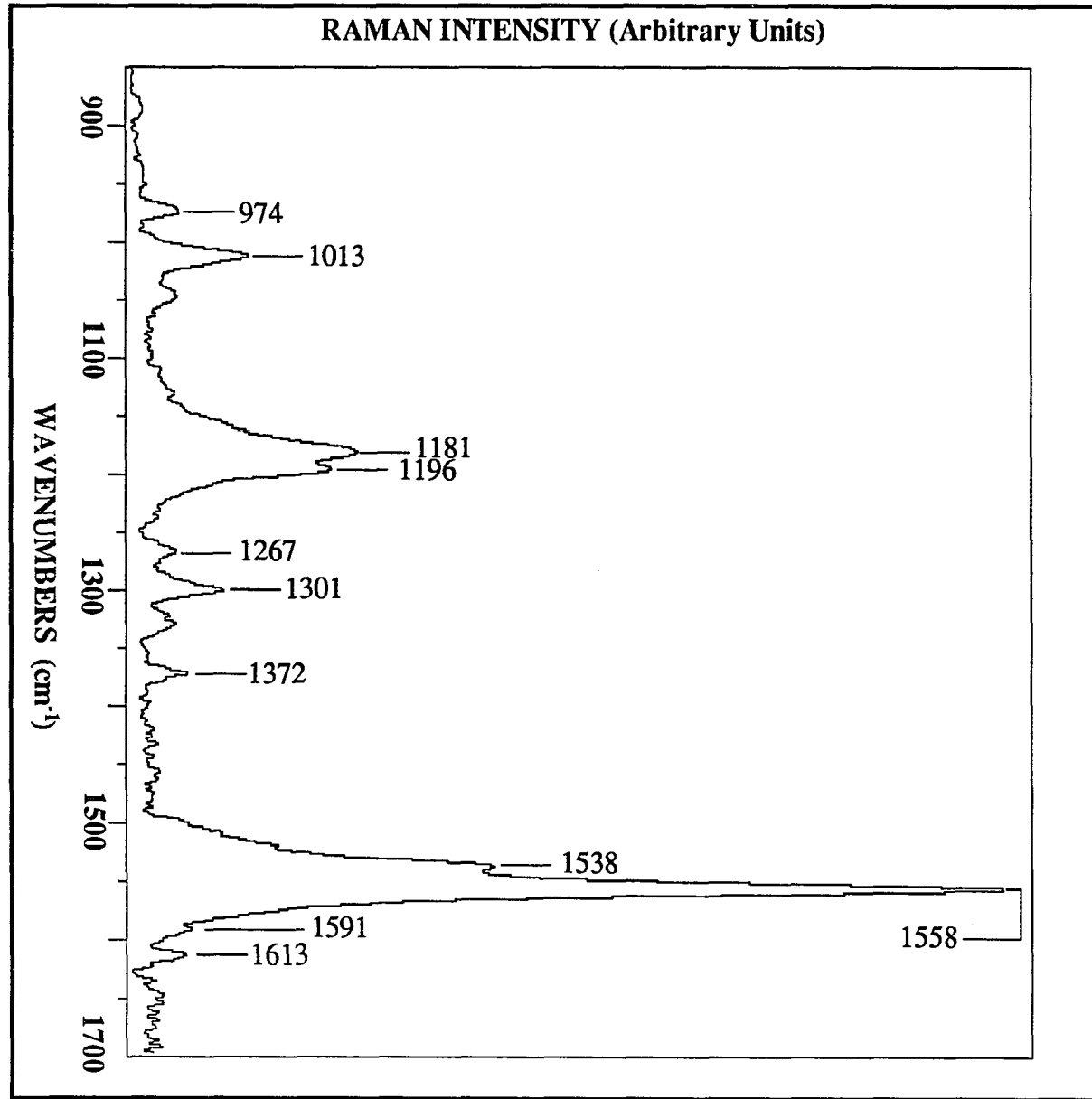


Figure 7.3

Figure 7.4 Raman spectrum representing a mixture of 13-bromo-bR and its L photo-intermediate from a rotating spinning cell at room temperature. The 514.5 nm laser line of an argon ion laser was used at the power level of ~100mW for Raman excitation. The photo alteration parameter was ~2.08 and the transit time of a molecule through the probe beam was 11 μ sec.

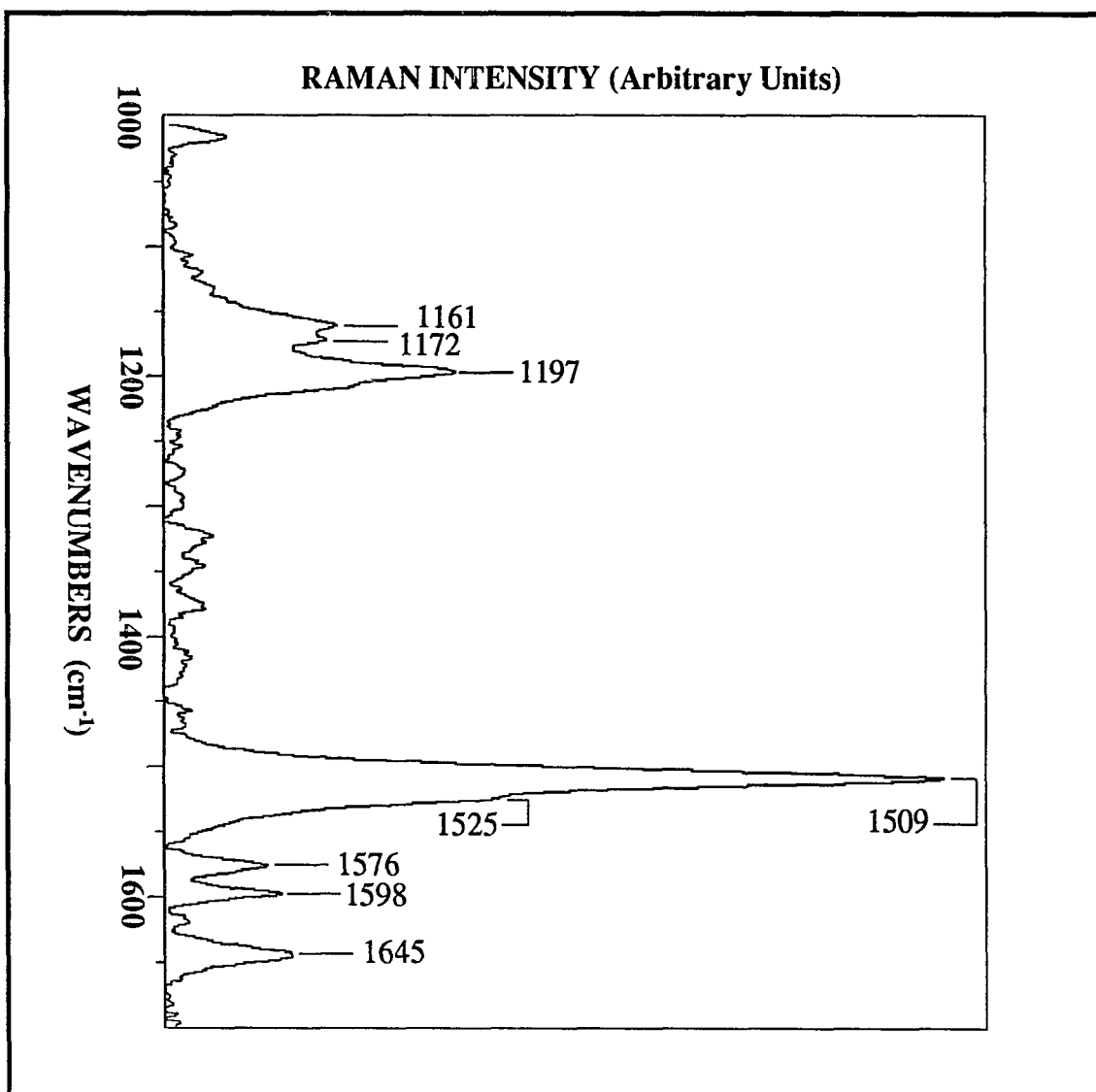


Figure 7.4

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