Downloaded from rsta.royalsocietypublishing.org

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A
MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

Resonance Raman Evidence for Secondary Protein--Schiff Base Interactions in Bacteriorhodopsin: Correlation of the Primary Excitation Mechanism with a Model for Proton Pumping and Visual Transduction

A. Lewis

Phil. Trans. R. Soc. Lond. A 1979 **293**, 315-327
doi: 10.1098/rsta.1979.0099

Email alerting service Receive free email alerts when new articles cite this article - sign up in the box at the top right-hand corner of the article or click **here**

To subscribe to *Phil. Trans. R. Soc. Lond. A* go to: **http://rsta.royalsocietypublishing.org/subscriptions**

This journal is © 1979 The Royal Society

Downloaded from rsta.royalsocietypublishing.org

MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES
THE ROYAL SOCIETY A
PHILOSOPHICAL TRANSACTIONS OF

Phil. Trans. R. Soc. Lond. A **293**, 315–327 (1979)
Printed in Great Britain

Resonance Raman evidence for secondary protein–Schiff base interactions in bacteriorhodopsin: correlation of the primary excitation mechanism with a model for proton pumping and visual transduction

By A. Lewis
School of Applied and Engineering Physics, Cornell University, Ithaca, New York 14853, *U.S.A.*

Resonance Raman spectra of bacteriorhodopsin (in various isotopically labelled and natural environments) and rhodopsins demonstrate that in all bacteriorhodopsin species with protonated Schiff base linkages, there is a secondary protein–Schiff base interaction. Steady state resonance Raman spectra of ^{15}N labelled purple membrane fragments and kinetic resonance Raman spectra (k.r.R.s.) as a function of pH, are consistent with the suggestion that the ϵ-amino group of a lysine residue is responsible for this secondary protein–protonated Schiff base interaction. Spectra of bacteriorhodopsin photo-intermediates with unprotonated Schiff base linkages demonstrate that the secondary protein–Schiff base interaction is absent in these species. These results, together with the accessibility of the protonated Schiff base in bacteriorhodopsin (bR_{570}) to solvent from the external medium and the insensitivity to pH of the secondary lysine–Schiff base complex in bR_{570}, suggest a structure for the complex in which the Schiff base proton is interacting with an amino acid side chain in an unprotonated configuration. In addition k.r.R.s. as a function of pH has demonstrated that the protein–Schiff base complex has a $pK > 12$ before light absorption and a pK in the range between 9.9 and 10.3 microseconds after light absorpion. These results suggest a molecular mechanism for proton pumping, which appears to account for the changes that are observed in the visible absorption spectrum and the resonance Raman spectrum as a function of proton pumping and pH. Finally, our results on bacteriorhodopsin and rhodopsin indicate that the primary excitation mechanism in these pigments, which produce remarkably similar absorption red shifts on similar time scales with different chromophore conformations and Schiff base interactions, must involve some region in the active site removed from the Schiff base and unrelated to a simple 11-*cis* to all-*trans* isomerization. A proton translocation in the protein together with retinal structural alteration (Lewis 1978) certainly fits all our observations and results in plausible molecular transformations which can account for the spectral similarities of all rhodopsins and bacteriorhodopsin while accounting for their functional diversity.

Introduction

This paper focuses on differences in the active sites of rhodopsin† and the purple membrane protein, bacteriorhodopsin,‡ which may be related to the widely differing biological roles of these two proteins.

The purple membrane protein was called bacteriorhodopsin because of its remarkable spectral and other similarities to rhodopsin. For example, it has been shown that absorption of a photon in both these proteins induces a *ca.* 45 nm red shift in the visible absorption of the retinal chromophore in < 6 ps (Busch *et al.* 1972; Kaufmann *et al.* 1976; Ippen *et al.* 1978). In addition, in all visual photoreceptor rhodopsins and bacteriorhodopsin this red shifted species is transformed thermally to an intermediate with an absorption maximum that is blue shifted (Yoshizawa & Horiuchi 1973; Lozier *et al.* 1975; Kung *et al.* 1975) and is approximately

†, ‡ For footnotes see following page.

The Royal Society is collaborating with JSTOR to digitize, preserve, and extend access to *Philosophical Transactions of the Royal Society of London. Series A, Mathematical and Physical Sciences.*
www.jstor.org

Downloaded from rsta.royalsocietypublishing.org

the same as the original pigment absorption. Furthermore, resonance Raman spectroscopy has demonstrated that the Schiff base in bacteriorhodopsin (Lewis *et al.* 1974), bovine (Lewis *et al.* 1973; Oseroff & Callender 1974) and squid (Sulkes *et al.* 1976) rhodopsins is protonated and can be deuterated.

This last result prompted investigators to model the chromophore of rhodopsin and bacteriorhodopsin with simple protonated Schiff bases of retinal, and initial experiments with squid (Sulkes *et al.* 1976) and bovine pigments (Mathies *et al.* 1977) yielded encouraging results. These experiments demonstrated that, although chromophore absorption in and out of the membrane differed by as much as 70 nm, the ground state resonance Raman vibrational frequencies of the pigments could be modelled very well with free simple protonated Schiff bases of retinal in the appropriate conformation. The results suggested that alterations in the chromophore absorption by the opsin matrix occurred as a result of chromophore excited state stabilization by charges in the opsin matrix. More recently, in a similar vein, Aton *et al.* (1977) attempted to model the resonance Raman spectrum of the chromophore of bacteriorhodopsin with the spectrum of a simple protonated Schiff base of all-*trans* retinal. This attempt was unsuccessful, and these workers attributed the differences they observed to deviations in the retinal structure from an all-*trans* conformation.

However, this conclusion of Aton *et al.* (1977) is not the only explanation for the failure of the above bacteriorhodopsin (bR_{570}) modelling experiments. It was implicitly assumed (Aton *et al.* 1977) that bR_{570} should be modelled by a simple protonated Schiff base model compound. However, the above assumption is questionable in view of the observation that the protonated Schiff base ($\text{—C=}\overset{\text{H}}{\underset{+}{\text{N}}}\text{—}$) stretching frequency in bacteriorhodopsin is at 1642 cm^{-1} whereas in visual pigments this vibrational mode occurs at *ca.* 1655 cm^{-1}, in very good agreement with data on model compounds of retinal complexed to protonated Schiff bases. This result takes on added significance when one considers (as discussed above) the other successes at modelling rhodopsin and failures at modelling bacteriorhodopsin resonance Raman spectra with simple protonated Schiff bases of retinal. Thus, these data suggested to us that, in addition to the observed differences in the isomeric configuration of the chromophore in rhodopsin (11-*cis*) (Wald 1968) and bacteriorhodopsin (all-*trans*) (Oesterhelt *et al.* 1973) there may also be differences in the nature of the Schiff base linkage in these two proteins.

---

† Rhodopsin, the primary light absorber and initiator of the visual process, is a quantum detector. It is composed of a polyene called retinal complexed via a Schiff base linkage (Bownds 1967) to an aqueous glycopolypeptide membrane matrix called opsin. After light absorption by the polyene chromophore, a series of thermal intermediates is produced with distinct absorption maxima (Yoshizawa & Horiuchi 1973). As demonstrated by biochemical extraction of the chromophore, these intermediates eventually transform the retinal from an initial 11-*cis* to a final all-*trans* conformation (Wald 1968).

‡ The purple membrane, which was discovered by Oesterhelt & Stoeckenius (Oesterhelt & Stoeckenius 1971) in the bacterium *Halobacterium halobium*, contains a single protein called bacteriorhodopsin because of its apparent similarities to rhodopsin. However, bacteriorhodopsin is not a quantum detector but an energy converter. It uses light energy to generate a proton gradient across the bacterial cell membrane. Like rhodopsin, this protein is composed of retinal complexed via a Schiff base linkage (Oesterhelt & Stoeckenius 1971) to an aqueous polypeptide membrane matrix called bacterio-opsin. Although after light absorption both rhodopsin and bacteriorhodopsin pass through a set of thermal transformations with remarkably similar absorption maxima (Lozier *et al.* 1975; Kung *et al.* 1975), biochemical extraction procedures demonstrate that the bacteriorhodopsin chromophore never achieves an 11-*cis* conformation and that the chromophore in bacteriorhodopsin is initially all-*trans* (Oesterhelt *et al.* 1973).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

Downloaded from rsta.royalsocietypublishing.org

MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A

In this paper we test this hypothesis with the use of kinetic and steady state resonance Raman spectroscopy on naturally occurring and isotopically labelled purple membrane fragments obtained from *Halobacterium halobium* grown in fully protonated, fully deuterated or ^{15}N enriched growth media. Our results demonstrate that a residue (in all probability a protein residue) in the bacterio-opsin matrix is interacting through the Schiff base proton with the chromophore Schiff base linkage. This secondary interaction with the protonated Schiff base is present in all protonated bacteriorhodopsin species and is absent in bacteriorhodopsin intermediates with unprotonated Schiff base linkages. In addition, the pH dependence of kinetic resonance Raman spectra is used to show that the protein–Schiff base complex has a pK > 12 before light absorption and a pK in the range between 9.9 and 10.3, microseconds after light absorption. These results clearly suggest a molecular mechanism for the proton pumping function of bacteriorhodopsin. Furthermore, in view of the very similar dynamic and spectral transformations observed in rhodopsin and bacteriorhodopsin primary photochemistry and in view of the fundamental differences we observe in the nature of the chromophore (isomeric configurations and Schiff base interactions), we conclude that the primary photochemistry is neither a change in the state of protonation of the Schiff base as has recently been suggested (Peters *et al.* 1977) nor an 11-*cis* to *trans* isomerization (Wald 1968) but, rather, light induced protein and retinal structural transformations involving in part a proton translocation in the protein matrix. Thus, our data support a mechansim of excitation (Lewis 1978) which not only accounts for all the photophysical and photochemical data on these proteins with a unique three dimensional energy surface (energy against protein deformation coordinate against retinal reaction coordinate) but also proposes a plausible molecular transformation which can readily initiate proton pumping in bacteriorhodopsin and can account for effective quantum detection and observations on loss of visual sensitivity in rhodopsin (Lewis 1978).

Materials and methods

Bacteriorhodopsin was isolated from *Halobacterium halobium* by the method of Kanner & Racker 1975). *Halobacterium halobium*, fully deuterated or 100% ^{15}N enriched, were grown at Argonne National Laboratories from a published procedure (Crespi 1977). The isotopically labelled purple membrane fragments isolated from these deuterated and ^{15}N enriched bacteria were the gift of Dr Henry Crespi, and our resonance Raman measurements indicated that they were better than 95% isotopically enriched. The fully protonated and isotopically labelled purple membrane fragments were then used to prepare H-retinal + H-bacterio-opsin, H-retinal + D-bacterio-opsin, D-retinal + H-bacterio-opsin, and D-retinal + D-bacterio-opsin as previously described (Marcus & Lewis 1977). Bovine rhodopsin was isolated from bovine retinas using a procedure described by Applebury *et al.* (1974). Model protonated Schiff bases of all-*trans* retinal were prepared with methylamine, ^{15}N methylamine, propylamine, butylamine, d_9 butylamine and hexylamine as reported previously (Marcus *et al.* 1978).

Steady state resonance Raman spectra were obtained with a Spex 1401 double mono chromator equipped with an RCA C31034 photomultiplier in a thermoelectric cooler and home-built photon counting electronics. All spectra were obtained in a stepping mode with 2 cm^{-1} steps, 5–10 second time constants and slits set for 2 cm^{-1} resolution. Spectra of the protonated Schiff bases and all kinetic resonance Raman spectra were obtained by using a flow apparatus (Marcus & Lewis 1977) and a spectrometer equipped with either a Spex

Downloaded from rsta.royalsocietypublishing.org

monochromator as described above or JY Ramanor HG2 double monochromator. Kinetic resonance Raman spectra were recorded by using the method of Marcus & Lewis (Marcus & Lewis 1977), and the pH of water suspensions of bacteriorhodopsin at a concentration of 2.8×10^{-5} M were adjusted with NaOH or HCl. The pH did not change during the kinetic measurements by more than 0.1, and thus we could avoid the use of buffers which at 0.03 M cause coagulation of the purple membrane fragments increasing the background scattering and noise.

Table 1. Isotopic effects on the Schiff base carbon–nitrogen stretching frequencies in bacteriorhodopsin suspended in H_2O and D_2O at pH 6.6. H = protonated, D = deuterated, ^{15}N = ^{15}N enriched

	—C=N— in H_2O or D_2O suspension (cm⁻¹)		C=N(H)+ in H_2O suspension (cm⁻¹)		C=N(D)+ in D_2O suspension (cm⁻¹)
H-retinal models	1622†		1655†‡		1630†
H-retinal + H-bacterio-opsin	1620	5	1642	7; 15	1620
H-retinal + D-bacterio-opsin	1618		1635		—
D-retinal + H-bacterio-opsin	1595		1625	5	—
D-retinal + D-bacterio-opsin	1594		1620	← 28 →	1592
H-retinal + H-bacterio-opsin (^{15}N)	1614		1627		1613

† Butylamine, d_9 butylamine, propylamine, and hexylamine all exhibit C=N(H)+ stretches at 1655 cm⁻¹ whereas methylamine and ^{15}N methylamine have similar shifts due to isotopic enrichment in the C=N and C=N(H)+ stretching vibrations.

‡ Data on model unprotonated and protonated Schiff bases were obtained by using ethanol as the solvent. Deuterated Schiff base data were obtained by using deuterated ethanol as the solvent. These model compound data are from Marcus *et al.* (1978).

Results and discussion

The —C=N(H)+— stretching frequency is the only vibrational mode in the resonance Raman spectrum of bacteriorhodopsin that is detectably affected by the isotopic nature of the protein–lipid (bacterio-opsin) matrix. The frequency of this mode in naturally occuring bacteriorhodopsin, in isotopically labelled bacteriorhodopsin and in model compounds is summarized in table 1 (column 2). From these data it is obvious that isotopical enrichment of bacterio-opsin with deuterium results in a lowering of the frequency of the —C=N(H)+— stretching mode by 7 cm⁻¹† and 5 cm⁻¹†. These shifts cannot be explained by deuterating the covalently linked lysine butyl side chain. This deduction is based on model compound data which demonstrates (see table 1, footnote †) that deuteration of the butyl group results in no shift in

† 7 cm⁻¹ for H-retinal in D- and H-bacterio-opsin and 5 cm⁻¹ for D-retinal in D- and H-bacterio-opsin.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

Downloaded from rsta.royalsocietypublishing.org

the $-\mathrm{C}{=}\overset{\mathrm{H}}{\underset{+}{\mathrm{N}}}-$ stretching frequency. In contrast, deuteration of bacterio-opsin has no effect on the unprotonated C=N stretching frequency. Therefore, these data indicate that there is, in bacteriorhodopsin species with protonated Schiff base linkages, a secondary lipid–protein interaction other than the covalently linked lysine side chain which affects the frequency of the $-\mathrm{C}{=}\overset{\mathrm{H}}{\underset{+}{\mathrm{N}}}-$ vibrational mode. The data also indicate that the interacting molecular species has non-exchangeable protons [as evidenced by our observation of a deuterium-induced shift in an H_2O environment], may have exchangeable protons [as evidenced by the observed 28 cm^{-1} downshift of the $-\mathrm{C}{=}\overset{\mathrm{H}}{\underset{+}{\mathrm{N}}}-$ stretching frequency of fully deuterated bacteriorhodopsin in H_2O and D_2O (table 1, columns 2 and 3) when compared to a predicted 24 cm^{-1} downshift if only the Schiff base proton is exchanged] and may have a nitrogen [as suggested by a complete review of our data on ^{15}N enrichment]. Furthermore, the loss of the interaction upon deprotonation of the Schiff base strongly suggests that the Schiff base proton is the link which establishes this secondary protein or lipid interaction at the Schiff base.

Identification of the interacting group

To identify the interacting group we have investigated, with the use of kinetic resonance Raman spectroscopy (k.r.R.s.), the dynamics of Schiff base deprotonation as a function of pH. Spectra at two extreme pHs show (figure 1) that the rate of Schiff base deprotonation (as detected by a band at 1620 cm^{-1}) is strongly affected by the pH of the external medium. In addition, titrating the appearance of this vibrational mode shows (figure 2) that the amino acid protonated Schiff base complex has a pK of *ca.* 9.9. The only nitrogen-containing amino acid residue with a pK in this vicinity is the ϵ-amino group of lysine or lipid components with this molecular configuration. In view of the reported lipid composition of the purple membrane (Kushwaha *et al.* 1976), we suggest but cannot unequivocally prove that lysine is the interacting molecular species. However, this suggestion makes the important but as yet unproved assumption that this pK corresponds to a relatively unperturbed group in the protein.

A proposed structure for the complex

To integrate the above data into a proposed structure for the interaction, two additional observations have to be considered. First, the immediate environment of the interaction is accessible to solvent from the external medium. This is demonstrated by the observation that the Schiff base proton can be exchanged for a deuteron (Lewis *et al.* 1974). Secondly, in spite of this accessibility, the interacting group can only be titrated microseconds after light absorption. This is indicated by the large proportion of species with $\mathrm{C}{=}\overset{\mathrm{H}}{\underset{+}{\mathrm{N}}}$ stretching frequencies at 1642 cm^{-1} even at pH 12 (figure 1*a*). Therefore, light must change the nature of the protein–protonated Schiff base interaction, activating it into a titratable species. The structure depicted

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

Downloaded from rsta.royalsocietypublishing.org

in figure 3*a* assumes for the sake of discussion that lysine is the interacting residue and meets the above criteria of solvent accessibility with pH insensitivity. Such a structure is by no means unique to bacteriorhodopsin; it has previously been proposed in other systems such as the deamination of α-methyl aspartic acid by the enzyme aspartate aminotransferase (Fasella *et al.* 1966).

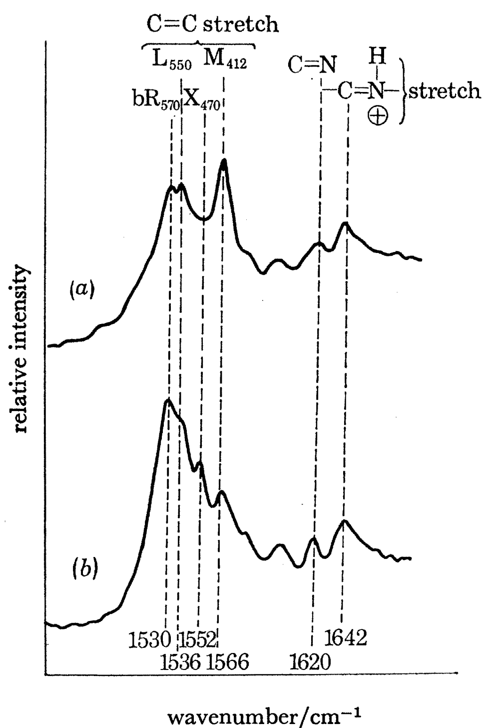

FIGURE 1

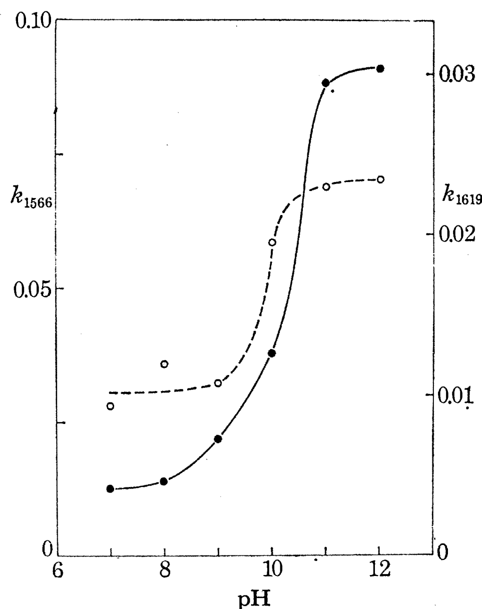

FIGURE 2

FIGURE 1. Kinetic resonance Raman spectra of bacteriorhodopsin at 6 μs and pH 12 (*a*) and 30 μs and pH 7 (*b*). These spectra were obtained with 457.9 nm laser excitation, and transit times in the laser beam of 6 μs and 30 μs respectively.

FIGURE 2. The formation rate constants as a function of pH for the 1620 cm^{-1} —C=N— stretching frequency (o– – –o) and the 1566 cm^{-1} M intermediate C=C stretching frequency (●——●). To obtain these titration curves the intensity ratios of the 1620 cm^{-1} C=N stretching frequency to the 1642 cm^{-1} —C=$\overset{+}{\underset{}{N}}$(H)— stretching frequency, and the 1566 cm^{-1} M C=C stretch to the 1530 cm^{-1} bR_{570} C=C stretch were plotted against time, and the initial slopes gave k_{1620} and k_{1566} respectively in units of μs^{-1}. The titration curves in this figure exhibit inflexion points at *ca.* 9.9 for k_{1620} and *ca* 10.35 for k_{1566}.

A previously undetected intermediate X: a possible pathway for complex dissociation

At pH > 9.9 (the p*K* of the activated protein–protonated Schiff base complex) it can be seen (figure 1*a*) that three C=C stretching frequencies are observed in the k.r.R.s. We show in figure 4 (open circles) that these stretching frequencies correlate well with the species observed on a timescale of several microseconds with transient absorption spectroscopy. This correlation is in agreement with previous suggestions that C=C stretching frequencies of retinylidene chromophores appear to be linearly correlated with the absorption maxima of these chromophores (Rimai *et al.* 1971). In view of the above results it is interesting that, at pHs below 9.9, a new C=C stretching frequency appears at 1552 cm^{-1} (see figure 1*b*). K.r.R.s.

Downloaded from rsta.royalsocietypublishing.org

demonstrates that this C=C stretching frequency arises from a species (we call X) which precedes M, follows L, and correlates (figure 4, filled circle) with an absorption maximum of between 460 and 480 nm. Earlier k.r.R.s. measurements at pH 7 (Marcus & Lewis 1977) strongly suggested that an unprotonated intermediate preceded M formation, and all our data on X are consistent with the suggestion that X is this unprotonated intermediate, previously

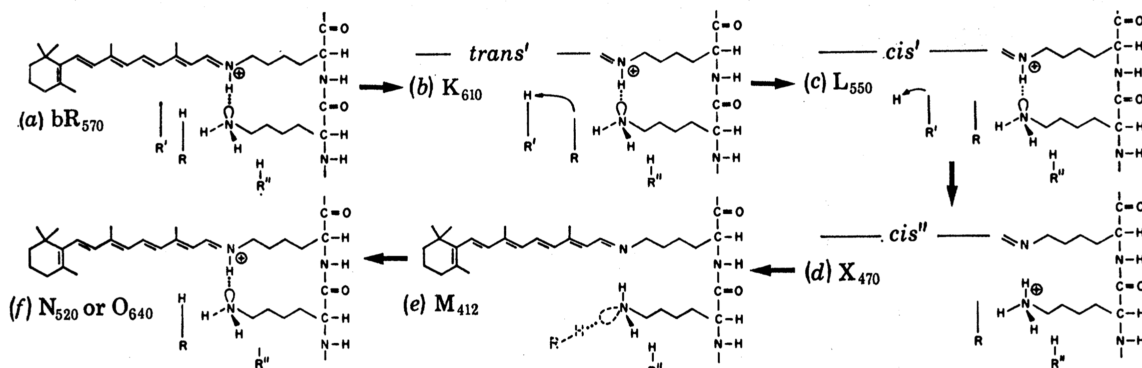

FIGURE 3. (*a*) Proposed structure for the protein–protonated Schiff base interaction. The amino acid sequence around the Schiff base in bacteriorhodopsin has been reported (Bridgen & Walker 1976; Ovchinnikov *et al.* 1977) and these investigations show that the sequence around the retinal lysine covalent linkage is very different in rhodopsin and bacteriorhodopsin. In bacteriorhodopsin there is a lysine residue adjacent to the lysine covalently linked to the retinal. In this figure we have tentatively used the ϵ-amino group of the adjacent non-covalently linked lysine as the interacting residue. However, until a detailed X-ray structure is available it will not be known if indeed this is the interacting protein residue or whether some other residue, which is close in space but removed in sequence, is responsible for the interaction. (*b*) A plausible molecular transformation in the protein for the photon induced bR_{570} to K_{610} transition. Such a transformation could readily initiate proton pumping and could be stabilized by some retinal structural alteration depicted as *trans'*. However, all indications are that this chromophore structural alteration does not include a *trans* to *cis* isomerization in the isoprenoid chain. (*c*)–(*f*) Plausible molecular transformations in the proton pumping cycle. This sequence is in agreement with the spectral data and assumes that X_{470} is not a side path entity but a precursor to M. It should also be pointed out that in K_{610} to X_{470} R will have to be in an environment protected from H_2O. In addition, the lysine —H—R complex in the M intermediate would prevent rapid reprotonation of the lysine residue in M at pH > 10.5. Chromophore conformational alterations are based on the kinetic and steady state resonance Raman results of Marcus & Lewis (1978) and are tentative at best. In addition, differences in the K and L, and L and X resonance Raman spectra may simply reflect changes in the state of protonation of the Schiff base rather than the chromophore structural alterations depicted in this figure. In order for proton pumping to occur the Schiff base will have to be reprotonated from the side of the membrane which is opposite to groups R and R′. The group responsible for reprotonation is depicted as R″ and is probably tyrosine (Lewis *et al.* 1978). This could occur directly or indirectly through the interacting lysine residue.

undetected by absorption spectroscopy. Thus, in summary, our data indicate that at pH > 9.9 (see figure 1*a*) the light activated protein–protonated Schiff base complex is rapidly deprotonated, forming the intermediate M with a C=C stretch at 1566 cm^{-1}. However, at pH < 9.9 (see figure 1*a*) this complex dissociation is slowed down sufficiently to reveal an intermediate state X with an unprotonated Schiff base and an absorption (between 460 and 480 nm) which is strongly perturbed from the 360 nm absorption of free unprotonated Schiff bases of all-*trans* retinal.

Assuming that X is not a side path entity, these results suggest that the appearance of the 1620 cm^{-1} unprotonated (C=N) stretch should be correlated with the L to X transition whereas the 1566 cm^{-1} C=C stretch of M should be correlated with the transition from unprotonated X to unprotonated M. Therefore, if the above hypothesis is correct the pH dependence of the 1620 cm^{-1} and 1566 cm^{-1} bands may be different and, as is observed in

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

Downloaded from rsta.royalsocietypublishing.org

figure 2, this appears to be the case. In fact the evolution of M exhibits a 1566 cm^{-1} titration curve which is not symmetric, indicating that it is composed of more than one pK. The overall pK of this titration curve is 10.3 which may even be slightly higher for the X to M transition alone. Thus, the pK of the X to M transition is in close agreement with the pK (10.5) of a relatively unperturbed lysine NH_3^+ ϵ-amino group. Although as previously noted the wide variations in the pK of amino acids in proteins do not allow us to make any assignment of the interacting residue based on these data alone.

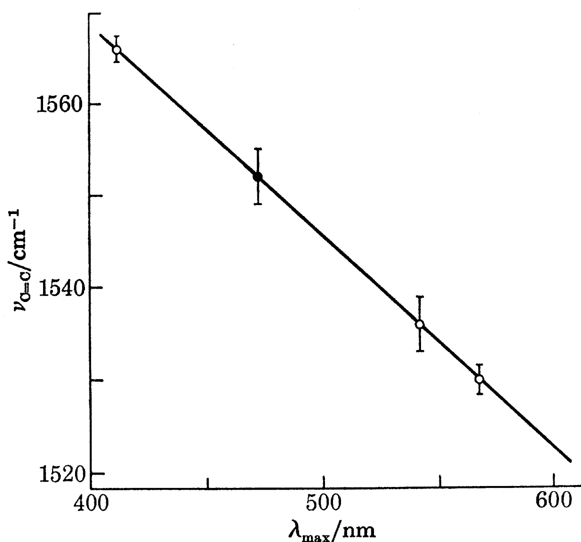

FIGURE 4. A plot of λ_{max} against $\nu_{C=C}$ for various intermediates in the proton pumping cycle.

A proposed molecular mechanism for proton pumping

In view of the deprotonation of the Schiff base between L and X, and the apparent different pH effects on the L to X and X to M transitions, we suggest that retinal and/or protein conformational changes between L and X alter the configuration of the protein–protonated Schiff base complex into a structure shown in figure 3*d* in which the absorption spectrum of the unprotonated Schiff base is strongly perturbed by the positive charge of the NH_3^+ group. Subsequently, deprotonation of the lysine NH_3^+ removes the strong positive charge perturbation at the Schiff base nitrogen and forms M_{412} as shown in figure 3*e*.

In this scheme it is difficult to observe X at high pHs because of rapid deprotonation of either L with a complex pK of *ca.* 9.9 (lowered from K by retinal and/or protein conformational changes) or X with a complex pK of $\geqslant 10.3$. This rapid deprotonation probably occurs as a result of competition from the external medium. However, at physiologically relevant pH (pH $\approx$ 7) for the lysine NH_3^+–unprotonated Schiff base complex in X to deprotonate and form M [as is observed (see figure 1*b*)], a molecular species with pK > 10.3 and lacking a proton must be present. Although we cannot exclude other possibilities, one candidate for this proton deficient high pK group (R in figure 3) is arginine, with a pK of *ca.* 12.5. This would account for our observations (see figure 1*a*) that photochemical cycling associated with proton pumping occurs up to pH 12. For arginine to lack a proton at pHs below 12.5, a large perturbation is required. Therefore we suggest that the photochemical transformation from bR_{570} to K by light is in part the translocation of a proton from R (arginine) to R′ (see figure 3*b*). One possible candidate for R′ is aspartic acid, and this is based on the observation (Oesterhelt & Stoeckenius

Downloaded from rsta.royalsocietypublishing.org

MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES
THE ROYAL SOCIETY A
PHILOSOPHICAL TRANSACTIONS OF

1971) that at pH 2.5 the absorption of bR_{570} red-shifts to 605 nm (*ca.* the 610 nm λ_{max} of *K*) and proton pumping stops. Furthermore, the timescale for the transition from K to L and the energy stored in the K intermediate (Lewis 1977; Rosenfeld *et al.* 1977) lend additional support to this hypothesis of a proton translocation from arginine to aspartic acid. The timescale for the decay of K to L is 2 μs, and this is in very good agreement with a value of 1 μs obtained experimentally for acetate group (R′) dissociation by Eigen & Schoen (1955). In addition, it has been recently suggested that the K intermediate stores part of the photon energy (Lewis 1977; Rosenfeld *et al.* 1977) and kinetic arguments suggest that *ca.* 54.4 kJ of energy is stored in this intermediate (Rosenfeld *et al.* 1977). If arginine and aspartic acid are indeed the groups involved in this light induced proton translocation, then a simple free energy expression predicts *ca.* 50.2 kJ of energy is stored in K which is in good agreement with the predictions of Rosenfeld *et al.* (1977) based on kinetic measurements.

The primary mechanism of excitation

Lewis (1978) has recently proposed a mechanism of excitation in bacteriorhodopsin and rhodopsin in which the primary action of light is to cause significant charge redistribution in the retinylidene chromophore which subsequently induces proton translocation from one amino acid to another and chromophore structural alteration. Not only do our above results on bacteriorhodopsin support this mechanism but also recent picosecond absorption spectroscopy on vertebrate rhodopsin (when reinterpreted in light of the resonance Raman data shown in figure 5) provide additional support for this mechanism of excitation. In these recent absorption measurements, Peters *et al.* (1977) found that the rate of bathorhodopsin production was decreased by seven times upon resuspension in D_2O. They interpreted this result as an indication that the Schiff base proton in vertebrate rhodopsin is complexed to an amino residue such as histidine, and changes its position as a function of chromophore light absorption. However, the data presented in this paper do not support this interpretation. Our results show that even though the Schiff base proton in bacteriorhodopsin is strongly complexed with an amino acid such as lysine, there is no evidence for such a strong interaction in bovine or squid rhodopsin. In fact, as pointed out earlier in this paper, resonance Raman spectra of squid and bovine visual pigments are modelled well by simple protonated Schiff bases of retinal. An example of this is seen in figure 5. Notice that the $C{=}\overset{\text{H}}{\underset{+}{\text{N}}}$ stretching frequency in these spectra of bovine photostationary mixtures of rhodopsin, isorhodopsin and bathorhodopsin is at 1655 cm^{-1}, in good agreement with the 1655 cm^{-1} $C{=}\overset{\text{H}}{\underset{+}{\text{N}}}$ stretching frequency observed in the spectra of model compounds (table 1; Sulkes *et al.* 1976; Mathies *et al.* 1977). In addition, as is seen by comparing figure 5*a* and *b*, the integrated area of the 1655 cm^{-1} band is unaltered when a 580 nm laser is used in figure 5*b* to reduce the bathorhodopsin concentration (which is monitored by the loss of the 1537 cm^{-1} bathorhodopsin C=C stretch). Thus, these results strongly suggest that the retinal lysine covalent linkage in rhodopsin is a protonated Schiff base which is not strongly perturbed and is not altered in going from rhodopsin or isorhodopsin to bathorhodopsin. Therefore, since the Schiff base is the only exchangeable group in the chromophore, the results of Peters *et al.* (1977) must be reinterpreted in terms of a proton translocation in the

Downloaded from rsta.royalsocietypublishing.org

opsin matrix in support of the excitation mechanism proposed by Lewis. Furthermore, additional support for this excitation mechanism can be found in the observation that, even though rhodopsin and bacteriorhodopsin have different Schiff base linkages and different chromophore isomeric configurations, these pigments still exhibit remarkably similar red shifts subsequent to photon absorption. Such an observation certainly does not support mechanisms of excitation based simply on isomerization or Schiff base protonation changes, and thus these results suggest that the mechanism of excitation in bacteriorhodopsin and rhodopsin is translocation of a proton in the protein together with retinal structural alteration (Lewis 1978).

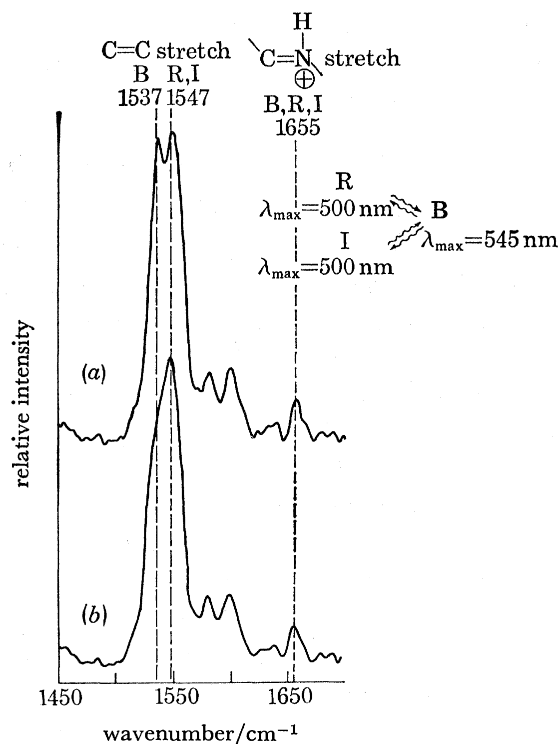

FIGURE 5. Resonance Raman spectra of photostationary mixtures of bovine rhodopsin (R), isorhodopsin (I), and bathorhodopsin (B). Spectrum (*a*) was obtained with 482.5 nm laser excitation. In spectrum (*b*) the probe beam was still at 482.5 nm; however, an additional 580 nm laser beam was used to pump the photochemistry. The addition of the 580 nm laser beam in spectrum (*b*) significantly reduces the bathorhodopsin concentration as is seen by the reduction of the 1537 cm^{-1} C=C stretch of bathorhodopsin. However, there is no change in the integrated area of the 1655 cm^{-1} —C=$\overset{H}{\underset{+}{N}}$— stretch in (*a*) and (*b*).

CONCLUSION

In conclusion, our results on rhodopsin and bacteriorhodopsin are summarized in figure 6. In the transition between R and R*, the chromophore in both these proteins can undergo significant charge redistribution which causes alternation of double and single bonds in the vertically excited state (R*). We have proposed (Lewis 1978) that the protein deforms as a result of this charge redistribution in the chromophore and generates a new protein structural state in P_X which stabilizes the chromophore charge redistribution. As we have noted (Lewis

Downloaded from rsta.royalsocietypublishing.org

1978), a likely protein structural alteration is the partial movement of a proton from one amino acid in the protein towards another, and this suggestion is supported by the data on bacteriorhodopsin presented in this paper. The next step in this excitation mechanism is torsional out-of-plane motion in the retinal [depicted by arrows in figure 6 (P_X)] which causes the excited protein–retinal complex in P_X to cross back onto the ground state surface producing after further ground state protein structural alteration the photochemical product Batho. Additional experimental verification for part of this excitation mechanism (ground state protein structural alteration in batho production) is contained in recent data published by Applebury *et al.* (1978)

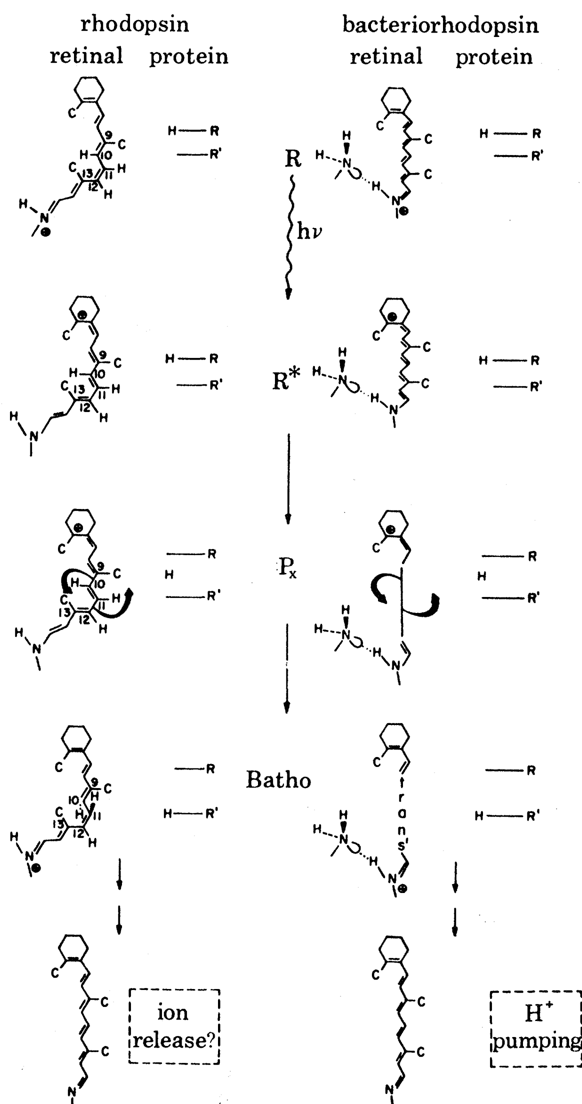

FIGURE 6. Protein and retinal structural alterations in rhodopsin and bacteriorhodopsin upon light absorption. Bacteriorhodopsin has interactions at the Schiff base which appear to be related to the proton pumping, energy transducing functions of this protein. Rhodopsin, on the other hand, may not have these Schiff base protein interactions since it is apparently not a proton pump (Hagins & Yoshikami 1977), but does exhibit conformational freedom which allows significant chromophore structural distortion, eventually leading to isomerization. This chromophore structural distortion appears to effectively stabilize the new Batho protein structural state induced by light, assuring the high quantum efficiency which is an essential aspect of the quantum detection function of rhodopsin.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

Downloaded from rsta.royalsocietypublishing.org

in which a deuterium isotope effect on bacteriorhodopsin batho evolution was observed at times that are long compared to the 1 ps ground state recovery detected by Ippen *et al.* (1978). This interpretation of the results of Applebury *et al.* (1978) in terms of the data of Ippen *et al.* (1978) is supported by coherent anti-Stokes Raman spectra of bacteriorhodopsin with high intensity laser pulses (Nelson *et al.* 1979). These spectra show none of the excited state effects which should have been observed if the excited state lifetime was as long as 10 ps (Applebury *et al.* 1978) rather than the 1 ps detected by Ippen *et al.* (1978). Thus, Applebury *et al.* (1978) were limited by their instrumental resolution ($\sim$ 6 ps) to measuring the ground state structural alteration we had suggested in our excitation mechanism (Lewis 1978) and this lack of resolution would also explain why these workers (Applebury *et al.* 1978) were unable to detect the ground state recovery of the initial bacteriorhodopsin species (bR_{570}) even though 70% of the excited state molecules return to the bR_{570} rather than the batho state.

In addition to the above, as is depicted in figure 6 (Batho) and as we suggested (Lewis 1978) retinal torsional motion in visual pigments generates a distorted chromophore conformation and this same torsional motion causes only minor structural alterations in the chromophore of bacteriorhodopsin. This could occur if the protein in bacteriorhodopsin lacks conformational freedom between carbon atoms 9 and 12, and some support for this hypothesis comes from the observation that visual pigments combine with 9 and 11-*cis* retinals whereas bacteriorhodopsin combines only with retinals which have similar conformations between C-9 and C-12 (such as the all-*trans* and 13-*cis* isomers). In addition, the data presented in this paper suggest that bacteriorhodopsin has a strong protein structural constraint at the Schiff base proton. This structural constraint should further restrict large chromophore structural alterations which would disrupt the functionally important protein–protonated Schiff base interaction.

Finally, the structural freedom in visual pigments and structural constraints in bacteriorhodopsin appear to be directly related to the biological roles of these two proteins. Rhodopsin is a quantum detector and in order to have a good quantum detector there must be molecular irreversibility. This is provided in the excitation mechanism depicted in figure 6 by retinal structural alteration in the R to Batho transition which stabilizes the new protein conformational state. In addition, the relaxation of this distorted chromophore structure to all-*trans* retinal further assures irreversibility by ensuring that the original rhodopsin structure will not be regenerated on the timescale of neural response generation. On the other hand, bacteriorhodopsin is an energy converter and, in order to have a good energy converter, endoenergetic conformational changes must be minimized and reversibility must be maximized. Thus the bacteria have evolved a restrictive molecular architecture which fulfils these fundamental aspects of an energy convertor and which is related to the proton pumping function of this protein. However, the retinal in this restrictive protein structure cannot stabilize the new protein conformation in Batho with the same efficiency as is displayed in rhodopsin and thus, the high quantum efficiency of visual photoreceptors, which is a fundamental aspect of a quantum detector, is sacrificed by more than a factor of two in bacteriorhodopsin.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

Downloaded from rsta.royalsocietypublishing.org

MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES
THE ROYAL SOCIETY
PHILOSOPHICAL TRANSACTIONS OF

References (Lewis)

Applebury, M. L., Peters, K. S. & Rentzepis, P. M. 1978 *Biophys. J.* **23**, 375–382.

Applebury, M. L., Zuckerman, O. M., Lamola, A. A. & Jovin, T. M. 1974 *Biochemistry* **13**, 3448–3455.

Aton, B., Doukas, A., Callender, R. H., Becher, B. & Ebrey, T. G. 1977 *Biochemistry* **16**, 2995–2999.

Bownds, D. 1967 *Nature, Lond.* **216**, 1178–118.

Bridgen, J. & Walker, I. D. 1976 *Biochemistry* **15**, 792–798.

Busch, G., Applebury, M., Lamola, A. & Rentzepis, P. 1972 *Proc. natn. Acad. Sci. U.S.A.* **69**, 2802–2806.

Crespi, H. 1977 *Proc. IAEA/CMEA. Tech. Committee, Meeting on Modern Trends in the Biological Application of Stable Isotopes*. Leipzig.

Eigen, M. & Schoen, J. 1955 *Z. Elektrochem.* **59**, 483–494.

Fasella, P., Giartosio, A. & Hammes. G. G. 1966 *Biochemistry* **5**, 197–202.

Hagins, W. A. & Yoshikami, S. 1977 *Biophys. J.* **17**, 196*a*.

Ippen, E., Shank, C. V., Lewis, A. & Marcus. M. 1978 *Science, N.Y.* (In the press.)

Kanner. B. & Racker, E. 1975 *Biochem biophys. Res. Commun.* **64**, 1054–1061.

Kaufmann, K. J., Rentzepis, P. M., Stoeckenius, W. & Lewis, A. 1976 *Biochem. biophys. Res. Commun.* **68**, 1109–1115.

Kung, M. C., Devault, D., Hess, B. & Oesterhelt, D. 1975 *Biophys. J.* **15**, 907–911.

Kushwaha, S. C., Kates, M. & Stoeckenius, W. 1976 *Biochem. biophys. Acta* **426**, 703–710.

Lewis, A. 1977 *Biophys. Struct. Mech.* **3**, 97–100.

Lewis, A. 1978 *Proc. natn. Acad. Sci. U.S.A.* **75**, 549–553.

Lewis, A., Fager, R. S. & Abrahamson, E. W. 1973 *J. Raman Spectrosc.* **1**, 465–470.

Lewis, A., Spoonhower, J., Bogomolni, R. A., Lozier, R. H. & Stoeckenius, W. 1974 *Proc. natn. Acad. Sci. U.S.A.* **71**, 4462–4466.

Lewis, A., Marcus, M., Ehrenberg, B. & Crespi, H. 1978 *Proc. natn. Acad. Sci. U.S.A.* **75**, 4642–4646.

Lozier, R. H., Bogomolni, R. A. & Stoeckenius, W. 1975 *Biophys. J.* **15**, 955–962.

Marcus, M. & Lewis, A. 1977 *Science, N.Y.* **195**, 1328–1330.

Marcus, M. & Lewis, A. 1978 *Biochemistry* **17**, 4722–4735.

Marcus, M., Lewis, A., Racker, E. & Crespi, H. 1977 *Biochem. biophys. Res. Commun.* **78**, 669–675.

Marcus, M., Lemley, A. T. & Lewis, A. 1978 *J. Raman Spectrosc.* **8**, 22–25.

Mathies, R., Freedman, T. B. & Stryer, L. 1977 *J. molec. Biol.* **109**, 367–372.

Nelson, E., Lewis, A. & MacFarlane, R. 1979 *Biophys. J.* **24**, 1179.

Oesterhelt, D., Meentzen, M. & Schumann, L. 1973 *Eur. J. Biochem.* **40**, 453–463.

Oesterhelt, D. & Stoeckenius, W. 1971 *Nature, new Biol.* **233**, 149–152

Oseroff, A. R. & Callender, R. H. 1974 *Biochemistry*, **13**, 4243–4248.

Ovchinnikov, Yu. A., Abdulaev, N. G., Feigina, M. Yu., Kiselev, A. V. & Lobanov, N. E. 1977 *FEBS Lett.* **84**, 1–4.

Peters, K., Applebury, M. L. & Rentzepis, P. M. 1977 *Proc. natn. Acad. Sci. U.S.A.* **74**, 3119–3123.

Rimai, L., Gill, D. & Parsons, J. L. 1971 *J. Am. chem. Soc.* **93**, 1353–1357.

Rosenfeld, T., Honig, B., Ottolenghi, M., Hurley, J. & Ebrey, T. G. 1977 *Pure appl. Chem.* **49**, 341–351.

Sulkes, M., Lewis, A., Lemley, A. & Cookingham, R. E. 1976 *Proc. natn. Acad. Sci. U.S.A.* **73**, 4266–4270.

Wald, G. 1968 *Science, N.Y.* **162**, 230–239.

Yoshizawa, T. & Horiuchi, S. 1973 In *Biochemistry and physiology of visual pigments* (ed. H. Langer), pp. 69–81. New York: Springer–Verlag.