Dynamic Processes in Visual Transduction

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I. Introduction

It is difficult to present to chemists a comprehensive view of a biological process such as vision: they are usually familiar only with the "molecular" language, while the overall process is presently treated in terms of a number of operational disciplinary languages. Most chemists accept as an article of faith that all facets of biological processes will eventually be expressible in a precise molecular language. To what degree this is possible has yet to be established. At the present time there is a considerable progress in this direction, as is evident from the increasing activity in the field termed "molecular biology".

We have tried to present here an overview of the elementary transduction process in vertebrate vision, i.e., the sequence of events beginning with the absorption of a photon of visible light and culminating in the generation of a hyperpolarizing electric potential in the photoreceptor cell. Obviously this elementary biological act is only a very small part of the overall process of vision but, in itself, it is very involved and, at present, can only be partially described in molecular terms. Accordingly, we have tried to organize our presentation so as to project the molecular theme into those areas where the prevailing disciplinary language is predominantly nonmolecular, e.g., electrophysiology.

In most vertebrate retinas two types of receptor cells can be distinguished: the rod cells (Figure 4) and the cone cells. The latter are responsible for color vision; the former function in black and white vision, usually at very low light levels, below the sensitivity threshold of the cone cells.

In this review we shall confine ourselves to a description of the dynamic processes in the dim light receptor cells, the rods. The main reason for this is that, at present, much more information is available on rods than on cones, the former outnumbering the latter by an order of magnitude in most species.

We shall first describe the phenomenology of the system, i.e., the input–output relationship of the photoreceptor. Next we shall list all the structural and known chemical units that constitute the receptor cell, and then we shall review the current picture of dynamic processes which occur in the cell, first in the dark, and then upon illumination. In the last section of this review we shall then discuss several hypothetical models of visual transduction, which try to link some of the described processes in a plausible sequence.

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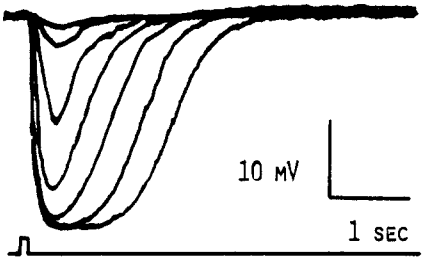


Figure 1. Superimposed traces of intracellularly recorded receptor potentials from the dark adapted receptor of *Gekko gekko*. The response increases with the intensity of the flash which, in turn, is increased in steps of 0.5 log units. Flash duration: 100 ms. Receptor cells are decoupled by aspartate treatment (adapted from Kleinschmidt[2]).

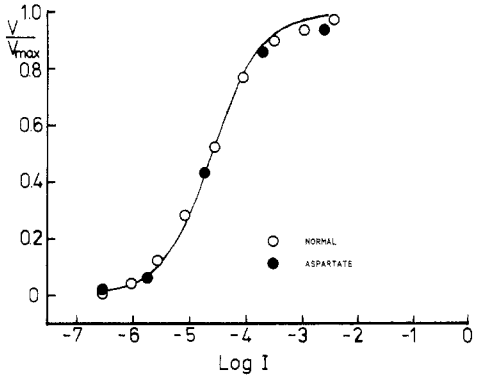


Figure 2. Response characteristics of the peak amplitudes of two dark adapted *Gekko* receptors, with and without aspartate decoupling. The solid line plots the hyperbolic tangent function $V/V_{max} = I/(I + \text{constant})$ (adapted from Kleinschmidt[2]).

II. Phenomenology

In this section we shall regard the receptor cell as a black box, describing its input–output relationship. The input is absorbed light intensity, i.e., a quantity that can be easily controlled. For measurement of the output, which is an electrical response, a microelectrode is inserted into the receptor cell. In the dark this electrode records a constant voltage of ca. −10 to −30 mV (depending on the species), relative to the outside of the cell. This value is roughly 20–50 mV more positive than the resting potential of most cells, which is determined mainly by the K^+ Nernst potential.[1]

Upon illumination, the receptor cell transiently hyperpolarizes; i.e., the membrane potential shifts toward the K^+-Nernst potential to form the so-called receptor potential, as illustrated in Figure 1. This change in membrane potential is thought to be the only means by which receptor cells react to illumination.[1,2]

The eye can be considered a quantum counter.[3] A single photon absorbed by the receptor cell can cause a measurable receptor potential. Up to a quantum density of ca. 30 quanta absorbed per flash per rod, the receptor potential increases linearly with light intensity, saturating at about 100–200 photons absorbed per rod.[4] Such a response characteristic is depicted in Figure 2. Thus, the *completely* dark-adapted eye not only can detect light at the quantum level but also can distinguish intensity changes in brief flashes of light over a range of more than two log units.

If the eye is exposed to a constant, steady illumination, a transient hyperpolarization of the receptor cell is followed by a partial return toward the original membrane potential. Thus the newly established resting potential is *below* that of the completely dark-adapted case. Under these conditions a sequence of increasing light intensity flashes again produces a hyperbolical response characteristic of the one in Figure 2 except that the dynamic range has been attenuated and the light intensity required for a given hyperpolarization, ΔV, has been increased.[4,5] This phenomenon, called *background adaptation*, expands the range of light intensity that a single photoreceptor cell can accommodate to about six orders of magnitude (Figure 3).

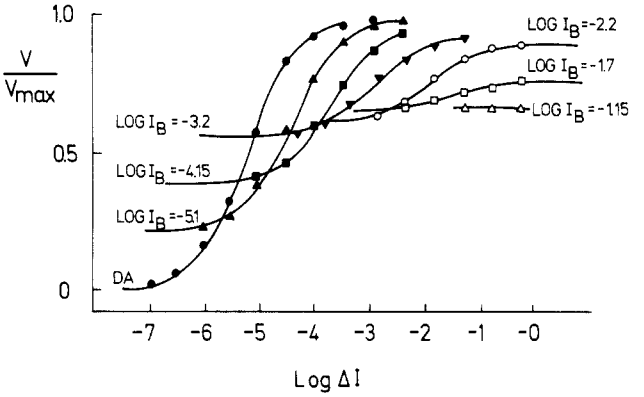


Figure 3. Background adaptation behavior of *Gekko gekko* photoreceptor cells, demonstrated as voltage intensity curves at increasing background light intensities. V is the flash-induced hyperpolarization and V_{max} the maximum hyperpolarization obtained in the dark adapted state (DA). Data were obtained from two cells (open and closed symbols) with slightly different V_{max} (after Kleinschmidt and Dowling[4]).

Considering the camera as an analogue of the eye, with the pupil corresponding to the aperture (the human pupil contributes an additional order of magnitude in adaptation) and the retina corresponding to the film, this would be equivalent to a film sensitivity that automatically adjusts its value over a range of 1 000 000 ASA or 60 DIN.

Background adaptation should be distinguished from another phenomenon called *dark adaptation*. When the vertebrate rod cell is exposed to light intensities that bleach an appreciable number of the pigment molecules, even without any background light, the sensitivity of the receptor cell is greatly reduced.[6,7] This drop in sensitivity far exceeds that expected simply on the basis of the reduced probability of photon capture by the partially bleached rod (pigment adaptation). The sensitivity recovers again in the dark, following a time course that appears to be related to pigment regeneration.[6,7] This phenomenon everyone experiences on coming from a bright to a dark environment. One's eyes need a few minutes in a dark room to recover their maximum sensitivity.

In summary, the receptor cell is not only a "quantum" detector that can translate the incoming light intensity information into an electrical response at the highest possible sensitivity but is also a device that can automatically control this sensitivity over a 6 log unit range of light intensity. In this way it can cope with an enormous variation of ambient light levels.

Until very recently it was thought that the major part of adaptation was accomplished not in the receptor cell but rather at the level of the neural retina. Now, however, it appears reasonably well established that the basic aspects of visual adaptation in the vertebrate retina originate largely in the receptor cells themselves.[4,5] In consequence, any hypothetical model of

TABLE I. Dimensions of Rod Cells[9-11]

species	length, μm	diameter, μm
human	44	1.8
necturus	60	12
frog	50	6
rat	24	1.7
cattle	10	1

visual transduction has to explain not only how single photons can be detected by the receptor cell but also the adaptation phenomena described above, which enable the photoreceptor cell to work at peak performance at widely differing ambient light levels.

III. Morphology

The rod photoreceptor cell is the part of the retina which absorbs incident photons and translates this light-intensity signal into the receptor potential.

Both morphologically and functionally, the rod cell is divided into two subunits: the outer segment (ROS), where the photopigment rhodopsin is located and where the transduction process takes place, and the inner segment (RIS), containing the nucleus, endoplasmic reticulum, Golgi complex, numerous mitochondria which energize the cell, and the synaptic terminus which synapes with the bipolar and horizontal cells of the retina.[8]

Our principal concern is with the outer segment, a cylindrical structure. Its dimensions vary considerably with different species, as shown in Table I.[9-11] In 1935 Schmidt noted a lamellar substructure in rods perpendicular to their longitudinal axis.[12] Under the electron microscope a stack of between 500 and 2000 flat, topologically closed membrane sacks, called "disks", can be discerned. The disks are separated from the extracellular medium by a plasma membrane which envelopes them.[13-15]

Only at the basal end of the outer segment, next to the inner segment, where disks are formed, the electron micrograph shows a morphological connection between disk and plasma membrane. Here, like in the outer segment of the cone photoreceptor, the disks are continuous with the plasma membrane, and the intradiskal space is identical with the extracellular one. From this it has been concluded that the disks form as invaginations of the plasma membrane.[16-18] However, electron microscopic studies of the bovine retina suggest that the rod disks arise directly from the cilium[19] and that both disks and plasma membrane form as evaginations of the cilium (Steinberg, personal communication).

In the majority of the disks, however, there appears to be no topological or electrical connection between disk and plasma membrane.[19-21] Schnetkamp, on the other hand, has recently provided solid evidence for a direct communication path between the disk interior and the extracellular fluid.[22]

Freeze–fracture electron microscopy as well as X-ray diffraction studies of the outer segment reveal the lamellar disk structure to be extremely regular, almost crystalline.[23-27] Worthington, for example, obtained as many as 20 diffraction maxima and minima from isolated frog outer segments.[25] The disk-to-disk repeat distance is 300 Å.[23-27]

Of the total outer segment membrane, only 1–3.5% is plasma membrane.[28] Presumably, the pigment molecule rhodopsin is contained in the plasma as well as the disk membrane. However, it is clear that the vast majority of the rhodopsin molecules are embedded in disk membranes.[29-31]

The disk membrane appears to be composed of a lipid bilayer. Embedded in this bilayer are the pigment molecules and the other membrane proteins, forming a membrane 60–70 Å thick. This membrane surrounds a small aqueous space (lumen), between 10 and 30 Å thick in its in vivo state. The cytoplasmic space between two adjacent disks is about 150 Å in thickness.[23-27,32,33]

The two topologically closed membrane systems in ROS, i.e., the disk membrane surrounding the disk lumen and the plasma membrane enclosing the disks and cytoplasm, constitute separate osmotic compartments. They both change in volume when the osmolarity of the bathing medium is changed.[34] It is interesting to note that both rods and disks seem to shrink and swell along a single dimension: rods become shorter or longer with changing osmotic pressure but do not change their diameter. Similarly, disks change only their thickness while retaining a constant radius.[11,35] Only when the osmolarity is very drastically reduced will rods lose their cylindrical shape and their disks become spherical.[34-36] This extraordinary behavior is attributed to the rim of the disks, which contains proteins not found elsewhere in the ROS[37] and that form a solid ring structure.

In addition to these two osmotic compartments, there is some evidence for the existence of a third, gellike matrix between the disks which appears to exhibit osmotic swelling and shrinking.[34] At present, however, very little is known about the composition of this interdiskal or its properties.

Under normal circumstances disk membranes are continuously renewed by the already mentioned cilium evagination at the basal end of the ROS. Simultaneously, the oldest disks are shed from the distal end of the outer segment, after which they are phagocytized and digested by pigment epithelium cells. This renewal cycle varies from a few days in the rat to a month in the frog. Dark exposure decreases the shedding rate while continuous high light levels cause a drastic increase in the rate, accompanied by an accelerated formation of new disks.[38-42] An interesting point in the context of the receptor cell renewal is the finding that there is a large birefringence in isolated ROS which shows a steep gradient from the basal to the distal end of the rod with maximum birefringence at the distal end.[43]

The inner and the outer segments of the receptor cells are connected by a narrow stalk of tubules called the cilium. From the evolutionary point of view the outer segment is an extended, specialized cilium. A number of cilial tubules extend inside of the receptor cell from the inner segment well into the outer segment.[1,8] Their specific functions have not yet been determined.

Detailed studies of rod outer segments have been possible because ROS can be easily isolated in high yield and excellent homogeneity. Gentle homogenization or shaking of retinas in a Ringer solution breaks the outer segment at the weakest point of the cell, the cilium. The plasma membrane reseals at the point of breakage.[44-47] Only in the case of the large and fragile frog photoreceptor does breakage occur at places other than the cilium; occasionally part of the inner segment separates from the retina together with the ROS.[48] After

the ROS are separated from the neural retina they can be further separated from mitochondria and red blood cell contamination by density gradient procedures.[44-47,49,50]

Intact, resealed rod outer segments have been obtained from frog[8,51-54] and cattle[47,49] retinas. An indication of their intactness was based on their ability to maintain an in vivo like physiology[51-53] as well as the impenetrability of the plasma membrane to a fluorescent dye.[54] In cattle ROS, where the dye penetration test fails,[47] the diffusion barrier formed by the plasma membrane to H^+, Ca^{2+}, and small molecules like ATP can be used as a test of an intact plasma membrane.[47]

Procedures to prepare apparently intact, isolated disks have also been described.[55] The disk preparation so obtained has been shown, by means of a light-scattering technique, to contain a very narrow size distribution of unbroken visicles.[36] In the course of this disk preparation procedure, which involves a hypotonic shock treatment, membrane components seem to either be lost or to deteriorate, since the disks are no longer impermeable to H^+ ions.[56]

Very recently, attempts to isolate whole, physiologically intact rod photoreceptor cells have been successful.[57] For further information on the morphology of the outer segment, the reader is referred to an excellent review by Cohen (1972).[34]

IV. Composition

A. Rhodopsin

Only absorbed radiation can affect matter. In rod photorecetor cells the molecule that absorbs visible light and is responsible for the primary processes in vision is the red colored pigment rhodopsin, the principal membrane protein of the disks.

1. The Rhodopsin Chromophore

Since no amino acids absorb visible light, the protein rhodopsin absorbs visible light by way of its attached prosthetic chromophore. All visual pigment chromophores are derived from two closely related polyene aldehydes, retinal (called in the older literature "retinene$_1$" or retinal$_1$) and 3-dehydroretinal (earlier referred to as "retinene$_2$" or retinal$_2$). Both chromophores occur in the 11-cis form in dark adapted pigments. The structures of these aldehydes are shown in Figure 5. The retinal derived pigments are called "rhodopsins" (from the Greek, meaning red appearance) while the less numerous 3-dehydroretinal-based pigments are called porphyropsins (purple appearance).[58-62] These polyene aldehydes form visual pigments by chemical bonding through a Schiff base linkage to an ϵ-amino group of a particular lysine residue in the glycoprotein moiety.[63-65] The apoprotein (without chromophore) is called opsin.

While it is reasonably well established that the chromophore of rhodopsins is the 11-*cis*-3-dehydroretinylidene group[66,67] (Figure 5), it remains to be established whether the Schiff base linkage in visual pigments is protonated (>C=NH^+–), unprotonated (>C=N–), or perhaps hydrogen bonded to some H donor groups.[68] This is a point of considerable significance as it not only determines the absorption spectrum of the pigment but also dictates the nature of the primary photochemical process which, in turn, initiates the transduction process.

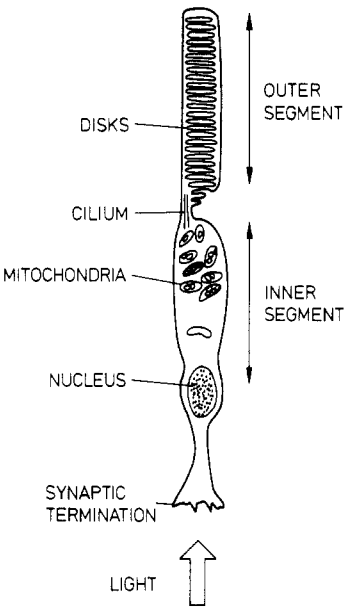


Figure 4. Schematic of the rod photoreceptor cell.

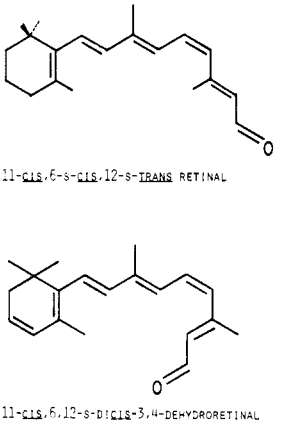


Figure 5. The two chromophoric bases from which all visual pigments are built. Rhodopsins use the base 11-*cis*-retinal and porphyropsins use the base 11-*cis*-3,4-dehydroretinal. Retinal and dehydroretinal are drawn in the two diffferent conformations, which could be present in native visual pigments.

The study of the chromophore structure and its detailed conformation in visual pigments is best pursued by physical techniques, preferably spectroscopic methods such as NMR, Raman, and, infrared, which probe the ground state. There are difficulties, however, in such spectroscopic studies because of interference from the glycoprotein moiety. Resonance Raman studies of bovine rhodopsin have been carried out in several laboratories,[69-72] and this technique has proved very useful because excitation in the rhodopsin absorption band essentially limits the scattered vibrational frequencies to the chromophore and to such groupings that may be closely coupled to it. A very recent study[73] of the infrared difference spectra of rhodopsin and its intermediates has also provided information relative to the chromophore. The results of the two methods, however, at the present time are in apparent disagreement as regards the question of the protonation of the Schiff base nitrogen atom.

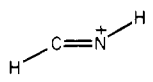
Perhaps the most potentially useful spectroscopic method for studying the chromophore of rhodopsin is

NMR spectroscopy. Interference by the glycoprotein moiety can, in principle, be minimized by using ^{13}C- and ^{2}D-enriched chromophores, but there are substantial problems in synthesizing such chromophores as well as preparing the labeled rhodopsin in sufficiently high concentration.[74] So far only a single successful NMR study of a ^{13}C-labeled chromophore in rhodopsin has been reported.[75] The results of this study, however, do not support the protonated Schiff base model of the chromophore on the basis of data from resonance Raman spectroscopy.[69-72]

Aside from the question of protonation there is the further question of the exact conformation of the chromophore. Examination of Figure 5 will show the model retinylidene chromophores such as *N*-retinylidenepropylimine (NRPI) and *N*-retinylidenepropyliminium ion ($NRPIH^+$) can exist in the s-cis or s-trans forms about the C_{12}–C_{13} single bond as well as in syn or anti forms relative to the Schiff base linkage. These possible conformations will be determined largely by the glycoprotein microenvironment of the chromophore. The microenvironment could also produce substantial torsions in the chromophore, particularly about single bonds. Its major effect, however, should be in controlling the absorption spectrum of the chromophore.

A number of studies of the ^{1}H[76-78] and ^{13}C NMR spectra[79,80] of isomers of retinals have been made, as these can be related to X-ray diffraction data from crystals.[81,82] Similar studies[74,83,84] on model Schiff bases of retinals are much fewer, as Schiff bases of pure isomers, particularly in protonated form, are very difficult to prepare.

^{1}H NMR spectral studies of the butylamine Schiff base of *all-trans*-retinal have been carried out by Sharma and Roels,[85] and very recently Tokito et al.[86] have reported ^{13}C NMR spectra of this compound. A rather extensive study of the ^{13}C NMR spectra of protonated and unprotonated propylamine Schiff bases of retinal isomers has been carried out in our laboratory by Dr. John Shriver.[74,83] This work has revealed a number of important structural aspects of these chromophores in solution as well as provided essential chemical shift data with which to compare the ^{13}C enriched chromophore in rhodopsin (see Figure 5). Of particular significance is the indication that protonation of the Schiff base nitrogen of ^{N}-(11-*cis*-retinylidene)-propylimine in $CDCl_3$ solution markedly alters the preferred conformation from a 12-s-cis to the torsionally distorted 12-c-trans forms (in crystals of 11-*cis*-retinal the 12-cis form is found exclusively[86]). This is borne out by both ^{13}C chemical shift data and ^{1}H nuclear Overhauser enhancement studies.[74] Furthermore, ^{13}C NMR studies with the shift reagent $Eu(fod)_3$ suggest that the preferred conformation relative to the Schiff base linkage is the anti form.



2. Spectra of Visual Pigments

A most striking feature of visual pigments which have so far been examined is the broad range of wavelength over which the spectral maxima occur. Considering that only two chromophores are known, this is truly remarkable. Pigments using the 11-*cis*-retinal base vary in long-wavelength absorption maximum (λ_{max}) from 345 to 575 nm while those based on 11-*cis*-3-dehydroretinal cover the λ_{max} range 338 to 620 nm[87] and possibly to 680 nm. What is apparent from this wide range of λ_{max} for each chromophore is that the glycoprotein microenvironment must provide means for a very substantial, yet precisely controlled, perturbation of the electronic structure of the chromophore.

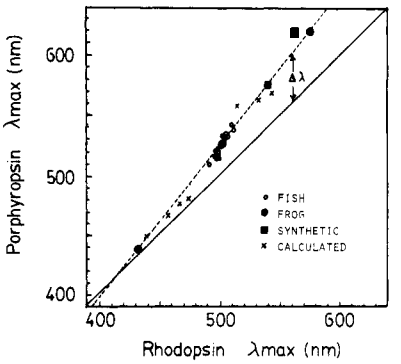


Figure 6. The Dartnall nomogram, showing the respective absorption maxima of rhodopsin/porphyropsin pairs which have the same apoproteins, i.e., opsins. Points designated by x are values of λ_{max} calculated by Cross[95] for the 11-*cis*, 6-*s*-*cis*, 12-*s*-*cis*-retinylidinium ion and 11-*cis*, 6-*s*-*cis*, 12-*s*-*cis*-3-dehydroretinylidinium ion in the field of a single negative point charge, using a modified Pariser–Parr–Pople LCAO–MO technique.

There is a class of fish known as Teleosts which, throughout the class, exhibit a wide range of λ_{max} for their visual pigments. Furthermore, they have a mixture of rhodopsin and porphyropsin in the same retina.[88] Wald et al.[89] have demonstrated that the same glycoprotein, "opsin", can form either a rhodopsin or porphyropsin, and this is presumably the case for the Teleosts whose rhodopsin/porphyropsin ratios vary seasonally while the total amount of pigment remains constant.[88] The absorption maxima of each rhodopsin–porphyropsin pair behave in the linear fashion shown in Figure 6.[88,89] It is apparent from Figure 6 that $\Delta\lambda_{max}$ increases toward the red, the region where most such pairs are found.

Several possible modes of spectral perturbation of the chromophore by the microenvironment have been suggested: (1) a Coulombic charged group distribution about the polyene chain;[90] (2) a dispersion interaction between the polyene chain and highly polarizable groups, e.g., aromatic amino acids;[91] and (3) torsion about selected bonds in the polyene chain.[92] Of the three modes, the one that has received the most attention theoretically has been the charge perturbation model. These studies have been recently reviewed by Honig and Ebrey[93] and by Suzuki.[94] Quite likely all three perturbation effects may contribute to the spectra of visual pigments. Charge perturbation, however, would seem to contribute in a major way as calculations by Cross on this model on both the retinylidene and 3-dehydroretinylidene chromophores fit the Dartnall experimental nomogram shown in Figure 6.

3. The Glycoprotein Rhodopsin

Until very recently it has been believed that the pigment rhodopsin overwhelmingly dominates the protein portion of the outer segment. It has been reported to constitute 85–90% of the total outer segment

protein.[96-98] This number, however, has recently come into question. Siebert et al. (1977) found that the so-called rhodopsin band on SDS gels from isolated ROS actually consists of three separate protein fractions only one of which appears to be rhodopsin.[99] Our laboratory has recently confirmed the resolution of these three fractions and furthermore demonstrated that only one of these three bands in the 40 000 molecular weight region is a glycoprotein, contains the retinylidene chromophore covalently bound in its native form, and is phosphorylated upon illumination. Therefore, only this fraction can be considered rhodopsin.[100]

Of the three (or more) protein components around 40 000 daltons, the two major ones are intrinsic disk membrane proteins since they cannot be washed out under any circumstances. According to the coomassie blue staining intensity, rhodopsin (apparent molecular weight 38 500) outweighs the other major protein (M_r 34 500) by a factor 2:1. Assuming that both proteins stain equally well with coomassie blue, rhodopsin would only account for ca. 60% of the total disk membrane protein.[100]

The existence of these additional proteins could explain why the lowest absorption ratio A_{280}/A_{500} found for single receptor cells, measured by means of microspectrophotometry, is 2.1–2.2[101] whereas purified rhodopsin shows a ratio of 1.5–1.6.[45] Since the absorption at 500 nm is solely due to rhodopsin, but less than 100% of the absorbance at 280 nm (in the case of purified rhodopsin) is due to the amino acids of the protein part of the pigment molecule,[102] the measured ratios of 2.1–2.2 and 1.5–1.6 also argue against the assumption that rhodopsin accounts for 90% of the ROS protein.

Rhodopsin is found in both plasma and disk membrane[103] and is embedded asymmetrically.[104-106] The carbohydrate chains of rhodopsins are found to protrude into the extracellular space in the case of the plasma membrane and into the intradiskal space in the case of the disk membrane. This indicates that neither during nor after the disk formation has a transmembrane flip-flop of the pigment molecule occurred.[107,108]

Dichroism studies have revealed that all the pigment chromophores in the disk membrane are oriented within 10° in one plane, parallel to the disk surface, i.e., perpendicular to the propagation direction of the incoming light,[101,109-111] affording the maximum chance of photon capture.

Within the plane of the disk membrane all possible orientations of the chromophore can be found. From the fact that linearly polarized light, entering the rod along its long axis, cannot induce any dichroism due to bleaching[112] unless the protein components of the disk membrane are cross-linked with glutaraldehyde,[113] it was concluded that rhodopsin can undergo rapid rotational diffusion in the plane of the disk membrane.[112,113] Cone determined the relaxation time for this process to be 3 μs at 20 °C.[114,115] Poo and Cone[116] and Liebman and Entine[117] showed that rhodopsin could also undergo translational diffusion. The values for the viscosity of the bilayer, calculated from the lateral and the rotational diffusion, were found to be in close agreement between *2 and 10 P*, indicating a very fluid membrane.

Rhodopsin in membrane-like environments appears to be highly asymmetric. Neutron scattering patterns from rhodopsin in a detergent micelle are consistent with an elongated pigment molecule, ca. 75–90 Å long.[118-120] This value is also obtained from X-ray diffraction[121] and fluorescence energy transfer studies[122] on rhodopsin micelles. Such an elongated rhodopsin molecule could easily span the disk membrane, and in fact structural evidence for a transmembrane rhodopsin has been obtained recently from X-ray,[121] neutron diffration,[118] and chemical labeling studies.[104,123-125] Functional evidence supporting this model comes from the work of one of us, R.U., who used the kinetics and the equilibrium constant of the metarhodopsin I–metarhodopsin II reaction (MI/II) as a membrane probe:[126] certain ions such as Ca^{2+}, Na^+, and H^+ have a different effect on MI/II, depending on whether they are in the extradiskal space only or also in the disk lumen. Thus, for example, cytoplasmic Ca^{2+} accelerates the MI/II reaction, whereas in the presence of Ca^{2+} and a suitable Ca^{2+} ionophore, the process is slowed down considerably. Since the ionophore itself has no effect on the kinetics, one may infer that the rhodopsin molecule communicates with both the extra- and intradiskal space.

Downer and Engländer[127] found from hydrogen–tritium exchange studies that about two-thirds of the peptide hydrogens in disk membranes are hydrogen bonded to water,[128] which is almost twice as much as in typical undenatured water-soluble proteins. One explanation for this may be that transmembrane rhodopsin is an integral part of a membrane "pore".

Rhodopsin is a single polypeptide chain with a molecular weight around 37 000.[28,102] Its presumed high α-helical content and polypeptide chain length are consistent with the possibility that as many as seven α-helical segments cross and recross the bilayer[120-124,129] in serpentine fashion. Digestion studies suggest it does so at least three times.[123,125,129-131] The C-terminal end of rhodopsin as well as the phosphorylation site are found to protrude into the cytoplasmic space while the N-terminal and the 11 sugar groups are found inside the disk.[107,130,131]

The chromophore of rhodopsin is close to the C-terminal end[130,131] and is embedded in a hydrophobic environment, as apparent from the fact that only the lipophilic sodium cyanoborohydride can reduce the chromophoric Schiff base bond in native rhodopsin while the hydrophilic sodium borohydride[132] cannot. Only when rhodopsin is photolyzed or denatured is the chromophore binding site exposed to the aqueous phase.[133]

The amino acid sequence of rhodopsin has only been partially determined. Hargrave[130,131] has analyzed the amino acids around the N-terminal with the carbohydrate binding side and around the C-terminal with its phosphorylation site. He finds that only serine and threonine are phosphorylated. The partial sequence in the vicinity of the chromophore is also known.[130,131] Successful isolation of the rhodopsin messenger RNA raises the hope that the whole amino acid sequence will soon be known.[134]

Rhodopsin has a somewhat larger than normal percentage of hydrophobic amino acids.[87,102] Determinations of the number of sulfhydryl groups have varied between 6 and 10,[28,135] and Dratz et al. recently reported that with very stringent protection from oxidation as

many as 12 sulfhydryls per rhodopsin can be found in ROS.[136] Applebury et al.[137] report that rhodopsin can be isolated as a detergent micelle in a completely lipid-free form and still retain its native absorption spectrum and normal sequence of intermediates. The CD spectrum of rhodopsin, however, is sensitive to the lipid and/or detergent environment.[138] Rhodopsin can be stabilized by the presence of lipids or particular detergents. The stability of rhodopsin in a particular detergent with regard to thermal denaturation[139] (which is highest in its disk membrane environment) and the extent to which bleached rhodopsin regenerates with 11-*cis*-retinal[139-141] were taken as criteria of detergent quality. According to these tests digitonin and octyl glucoside are the mildest detergents in which rhodopsin assumes a conformation closest to its native one.[139] Aside from rhodopsin, not very much is known about any other protein component of the ROS. Several enzyme systems associated with ROS have been found and characterized. They will be described in section VI of this review.

B. Lipids

The second major group of membrane components of ROS are lipids. Five phospholipids, phosphatidylcholine (40%), phosphatidylethanolamine (38%), phosphatidylserine (13%), sphingomyelin (3%), and phosphatidylinositol (2%), make up more than 95% of the total lipid content.[28,65,87] The cholesterol content of the ROS is remarkably low.[28,87] This, together with the extremely high degree of unsaturation of the phospholipids[142] (over 80% of them are unsaturated), gives the disk membrane the high fluidity required for the observed diffusional freedom.

The high polyunsaturated fatty acid content of the disk membrane makes it also very susceptible to lipid peroxidation.[143,144] In the intact system oxidative damage is prevented by vitamin E (α-tocopherol), which is present in relatively high concentrations in the ROS.[143,144]

The lipid composition of the ROS seems to be very critical. Animals fed with a diet lacking certain of the disk membrane phospholipids slow down their disk-renewing process and retain their original lipid composition at a time when in most other tissues, such as brain, heart, and kidney, it has already drastically changed.[145-147]

Since it has, as yet, been impossible to isolate the plasma membrane, it is not known whether its lipid composition differs from that of the disks. However, it has been established that the lipids of the disk membrane are asymmetrically arranged. Phosphatidylserine (PS) and phosphatidylcholine (PC) appear to be preferentially distributed on the inner surface of the bilayer, phosphatidylethanolamine (PE) on the outer surface of the disk.[124,148]

As to the ratio of rhodopsin to lipid molecules, recent data by Dratz and co-workers indicate that there are about 65 phospholipids per rhodopsin in the outer segment, two diglycerides, 5 free fatty acids, and 9 cholesterols.[124,148] They occupy an area of ca. 2580 Å^2 per rhodopsin molecule in the disk membrane, leaving ca. 800–900 Å^2 for the area occupied directly by rhodopsin.[148] The latter figure was derived from the known rhodopsin concentration in the outer segment (3.3 nM[101]) and from the disk to disk repeat distance of ca. 295 Å,[23-27] which, taken together, indicate a rhodopsin density of 1 rhodopsin per 3420 Å^2 of disk surface area. Thus only ca. 75% of the disk membrane surface is occupied by lipids, the rest by rhodopsin and other proteins. Therefore, the typical Singer model of a lipid bilayer membrane, where a few proteins "swim" in a sea of lipids, is, in the case of the disk membrane, misleading.

C. Carbohydrates

About 4% of the dry weight of the ROS is carbohydrate.[28] Of this, 25% can be attributed to be hydrophilic carbohydrate chains attached to the β-carboxyl group of aspartic acid in the 2 and 15 position from the N-terminal end of the amino acid chain of rhodopsin.[130,131,149,150] Staining with acridine orange indicates a considerable mucopolysaccharide concentration in the space between disk rim and plasma membrane.[48] As these mucopolysaccharides carry a high negative charge, they could conceivably play a role in the transport of transmitter ions between disk and plasma membrane.

V. Dynamics of the Dark-Adapted Receptor Cell

A. Dark Current

As already mentioned in section II, there is a membrane potential across the plasma membrane of the receptor cell differing from the usual K^+ resting potential of most cells. This difference arises from a current which flows externally in the dark from the inner to the outer segment.[151,152] It enters the outer segment envelope as an influx of ca. 10^9 sodium ions per rod per second.[153-157] A ouabain sensitive Na^+/K^+ ATPase, presumably located in the inner segment, pumps Na^+ out and K^+ into the cell, providing the ion gradients required to maintain the dark current. Poisoning this pump with cyanide completely abolishes the dark current within less than 1 min,[153] suggesting that a complete cation turnover takes place in less than this time.[151]

The Na^+ permeability of the outer segment is high and its K^+ permeability low,[51,119,153,158] while the reverse is true for the inner segment.[159] This raises the possibility that a K^+ current, entering the receptor cell through the pump sites and passively flowing out of the inner segment along its electrochemical gradient, forms an additional current loop.[51,160] This is shown in the schematic drawing of the electric circuits and ionic fluxes (Figure 7) that are thought to underlie the dark current in vertebrate rods.

The fact that the membrane potential of the receptor cell is markedly influenced by the Na^+ dark current, has suggested that the observed hyperpolarization of the receptor cell upon illumination is due to a decrease in this current.[51,96,153-158] It has been assumed that this Na^+ influx into the outer segment is reduced by a light-induced Na^+ permeability decrease[51,158,159] and that the means by which the receptor cell responds to light is by this light-dependent Na^+ permeability change.

B. Enzymatic Activities

Various enzymatic activities have been reported for the dark adapted receptor cell.

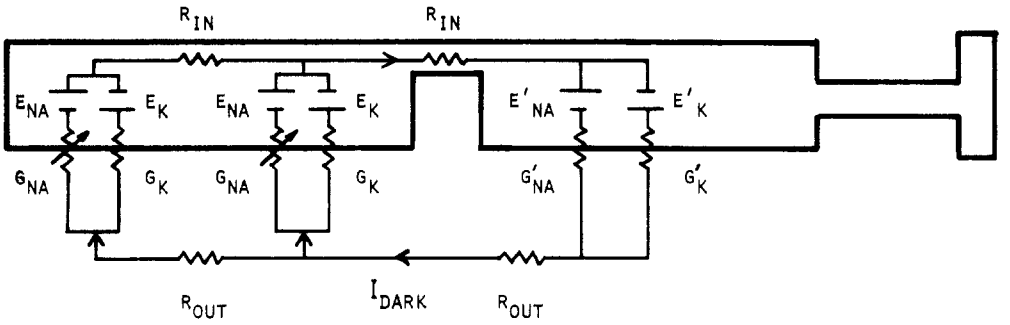


Figure 7. Simplified equivalent electric circuit for the entire rod cell (after Ebrey and Honig[102]).

1. Nucleotide Triphosphatases

A Ca^{2+}ATPase,[161-163] two Mg^{2+}ATPases,[164,165] and a GTPase[166-168] have been identified in the outer segment of the receptor cell. Their possible roles in the visual process, however, are not known. The Ca^{2+}ATPase could possibly account for the ATP-dependent Ca^{2+} uptake of disk vesicles or intact disks reported by Mason et al.[169] and Schnetkamp et al.[170]

The Mg^{2+}ATPases of Thacher[164] and of Uhl et al.,[165] both residing in the disk membrane, appear to be different enzymes, since only one of them[164] can also use GTP as a substrate. The other ATPase found in our laboratory is completely inhibited by DCCD (*N,N'*-dicyclohecylcarbodiimide), vanadate, and quercetin. Its action in the dark-adapted disk membrane is accompanied by a very pronounced decrease in light-scattering (initial rate: 10–20% per min) probably arising from a change in refractive index of the disk membrane.[171]

2. Kinases

There is a cyclic nucleotide-dependent phosphorylation of a 31 000 molecular weight protein in cattle and rats[172] and a phosphorylation of three low molecular weight polypeptides (12 000, 13 000, and 13 500 daltons) in frogs.[125] Their possible importance for transduction will be discussed in the next section.

3. Guanyl-cyclase Activity

High cGMP levels are maintained in the outer segment by a cyclase which is located in the cilium.[173] Low Ca^{2+} (in the presence of exogenous EGTA) increases both cyclase activity and intracellular cGMP levels 15–20-fold,[173,174] whereas high Ca^{2+} appears to inhibit the enzyme.[125]

C. Ca^{2+} Translocation and Storage in Isolated ROS

Schnetkamp has recently reported that in isolated, intact rod outer segment Ca^{2+} is predominately stored by binding at intradiskal binding sites.[175] The intradiskal membrane phase behaves as a cation-exchange system at which Ca^{2+} ions and protons can be reversibly exchanged. Moreover, there exists an endogenous cation-exchange system which makes the intracellular Ca^{2+} ions rapidly accessible to the extracellular space (even in the presence of an intact and impermeable plasma membrane) by means of a predominantly electronical exchange transport.[22] This transport system mediates Ca^{2+}–Ca^{2+} and Ca^{2+}–Na^{+} exchange and absolutely discriminates between Na^{+} and Li^{+} or K^{+}.[22,175]

D. GTP Binding Proteins

The two major soluble ROS proteins (M_r 37 000 and 41 000) bind GTP with high specificity and a high binding constant (K_b = 0.5 μM).[176] It is only when they have bound GTP that they can be readily extracted from disk membrane preparations. In the absence of GTP they appear to be membrane bound.[176] There is GTPase activity associated with these proteins.[176]

E. Regeneration of Rhodopsin

The regeneration of frog rhodopsin both in solution and in the isolated retina was observed a century ago by Ewald and Kühne.[177] The in vitro kinetics of the reaction in solutions of photobleached extracts were first studied by Chase and Smith[178] who found them to be second order, becoming first order at high concentrations of the glycoprotein moiety. Further studies using the physiologically active isomer 11-*cis*-retinal have shown that it will react with the glycoprotein moiety only if the latter is present in ROS[179,180] or when solubilized as detergent micelles in digitonin,[181] Tween-80,[182] octyl glucoside,[183] or sodium cholate.[184] In other detergents often used for solubilizing rhodopsin from ROS, i.e., Triton-X-100,[185] cetyltrimethylammonium bromide,[186] dimethyl laurylamine *N*-oxide,[187] emulphogene BC 720,[188] and dodecyltrimethylammonium bromide,[189] no rhodopsin formation could be observed.

Henselman and Cusanovich[189] have recently studied the kinetics of the reaction of 11-*cis*-retinal with the glycoprotein moiety in bovine ROS and in aqueous sodium cholate micelles. They find the reaction to be acid–base catalyzed, involving the usual intermediates postulated in model Schiff base formation. Initially, a second-order formation of an additional complex is postulated, followed by a first-order dehydration step to form a protonated Schiff base. Zorn and Futterman[182] and Shichi[190] find that phospholipids accelerate rhodopsin regeneration in detergent-extracted and delipidated preparations. The mechanism, however, for such a phospholipid-mediated synthesis of rhodopsin has not been elucidated.

The course of regeneration of rhodopsin in situ is a much more complicated problem since provision must be made for isomerization of the *trans*-retinal released by photolysis. In dark adaption in the frog the pigment epithelium plays a predominant role.[191-195] Hubbard and Dowling[195] have shown that retinal is stored here in the form of retinyl esters of fatty acids. There has

been some question as to the location of the "isomerase" which effects the conversion of the *all-trans*-retinal to the 11-cis form.[196,197] Bridges,[198] on the basis of extensive studies, proposes that in the frog the *all-trans*-retinal released on photolysis is reduced to retinol by NADPH and then migrates to the pigment epithelium where it is converted to retinyl ester and stored in oil droplets. Presumably in the form of retinyl ester, it is fed back to the ROS where it is converted via the isomerase to the 11-cis form. After hydrolysis of the ester, 11-*cis*-retinol is oxidized by NADP to 11-*cis*-retinal, which recombines with the apoprotein to form rhodopsin. The primary role of the pigment epithelium according to this scheme appears to be one of conversion of retinal to a fatty acid ester on which the isomerase can act.

In the isolated retina in the absence of the pigment epithelium, Sickel and co-workers[199] found that complete regeneration can occur when not more than 2.5% of the rhodopsin initially present is photolyzed. However, the cycle of photobleaching at this level followed by regeneration could be repeated well beyond the limit of any stored chromophores. Furthermore, they found the regeneration rate to be first order and very rapid (k = 0.12 min^{-1}). Under these conditions they concluded that this pathway for regeneration probably does not require 11-*cis*-retinal but more likely involves early intermediates in the photolysis cycle, not later than metarhodopsin II. It would appear, therefore, that only in the case of intense photobleaching where the capacity of internal regeneration system is exceeded does hydrolysis of the chromophore and exchange of retinol with the pigment epithelium occur.

VI. Light-Induced Dynamics of the Receptor Cell

The study of rapid physiological processes requires the use of rapid relaxation techniques. Furthermore, elaborate purification of biological material, the usual chemical procedure to assure accurate and precise results, yields data which differ markedly from corresponding in vivo studies. For this reason, most of our present knowledge on rapid, light-induced processes in the photoreceptor cell is a compromise between specificity and accuracy on one hand and physiological fidelity on the other.

Before the various light responses within the receptor cell are described in detail, we shall first list the techniques applied to obtain this information.

A. Techniques for Studying the Photoreceptor Dynamics

(1) Extracellular application of certain ions or reagents can affect the cell interior in a specific way.[51,153,200-204] One can introduce ions into the interview of cells or organelles by the vesicle fusion technique,[205,206] first applied to the study of visual transduction by Hagins and Yoshikami.[207] This technique has reduced the uncertainty of the site of action of various perturbations considerably. In this method ions or ion chelators[207] or specific enzyme inhibitors are trapped in lipid vesicles and carried into the cell by fusion of the vesicles with the plasma membrane. Their effect on the receptor potential can then be studied.

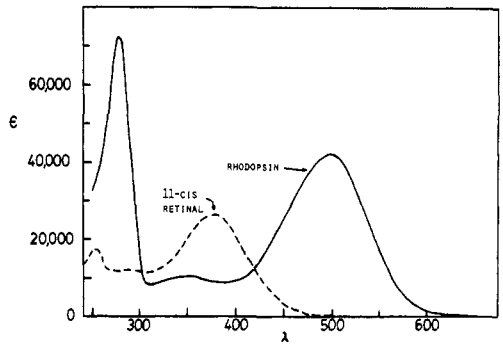


Figure 8. Absorption spectra of the chromophore of rhodopsin, 11-*cis*-retinal, and rhodopsin itself.

(2) The original light-induced transduction cascade can be initiated in an intact retina or isolated receptor cell by a flash of light and then, after precise short time invervals, stopped by rapid freezing techniques.[208,209] Similarly, certain biochemical reactions can be halted by acid quenching or rapid denaturation.[210] In these ways rapid light-induced changes in the concentration of certain substrate molecules can be monitored.

There are a variety of probes that can be used in the receptor cell in order to monitor rapid light-induced processes. Some of them involve natural components of the receptor cell, while others, artificial ones, can be introduced exogenously.

(3) *Natural Probes.* (a) The rhodopsin chromophore. The spectrum of the visual pigment rhodopsin, discussed in section IV, is quite distinct from the spectrum of the isolated pigment chromophore, 11-*cis*-retinal (Figure 8). Therefore, the rhodopsin spectrum can be taken as a probe for the perturbation of the electronic states of the chromophore by its protein environment. Light-induced processes change the microenvironment of the chromophore and, simultaneously, the perturbation can be readily identified and monitored as accompanying spectral changes. Low-temperature spectroscopy, flash photolysis, and recently laser flash photolysis have been applied to such studies. At this point, however, a word of caution relative to electronic absorption spectra appears to be in order: It could be misleading to focus exclusively on such spectrally observable intermediates of the rhodopsin chromophore when trying to correlate processes on the cellular or subcellular level with light-induced processes involving the entire pigment rhodopsin molecule, since it cannot be assumed that every conformational change in the protein moiety is reflected in the spectrum of the chromophore.

(b) The resonance-enhanced Raman scattering spectrum of the visual pigment chromophore can also be used as an internal probe. Again, only information concerning the chromophore and its microenvironment can be obtained.

(c) Spectroscopic techniques need not be confined to the rhodopsin chromophore: Raman, IR, and NMR spectroscopy use spectroscopic properties of molecules, including the nonchromophoric part of rhodopsin that comprises the receptor cell as natural probes. All three methods provide a large amount of data, often very difficult to interpret in molecular terms. Experience has been that the more intact the fraction of the receptor cell that is examined, the more complex will be the observed spectrum and the more difficult it will be

to interpret. Another obstacle is the poor time resolution of the above techniques. Siebert et al.,[73] however, have recently reported the first flash photolysis IR results from isolated bovine ROS with a time resolution of a few milliseconds. Thus, of the many IR absorption bands of the ROS, only those which undergo rapid light-induced changes are selected and studied. This simplifies the interpretation process considerably.

(4) *Artificial Probes.* Optical methods—pH, cation, and electric field sensitive dyes—can be used to measure ion uptake, release, or translocation in the ROS. Spin-labels can be applied in order to monitor lipid fluidity, lipid/lipid, and lipid/protein interaction, surface and transmembrane potentials.[211-213]

(5) Structural parameters can be used as a probe for transduction processes. The structure and ultrastructure of the ROS, as defined and depicted by means of microscopy (light and electron) and X-ray and neutron diffraction as well as light scattering can be utilized as another probe for light-induced transduction processes.

X-ray diffraction is limited by poor spatial and temporal resolution. X-ray exposure times sufficient to produce interpretable diffraction parameters have been reduced to about 30 s,[26,214] which is still 2 to 3 orders of magnitude slower than transduction and also involves considerable physiological perturbation. On the other hand, it does provide rather precise information as to what structural parameter actually changes upon illumination.

Light scattering[126,165,171,215-226] and birefringence measurements[227] (the latter can even be carried out under the light microscope on single cells) not only offer a much reduced perturbation of the observed system but their time resolution can also be relatively easily extended to microseconds. By means of volume measurement of the disks (light scattering)[56,126,228] and of the entire ROS (light scattering,[229] light microscopy,[51,158] Coulter counter[52]), permeabilities of these particular membrane systems can be measured as well as light-induced changes of these permeabilities. Birefringence changes can reflect increasing lipid order or disorder[227] or volume[230] and/or membrane potential changes.[231,232]

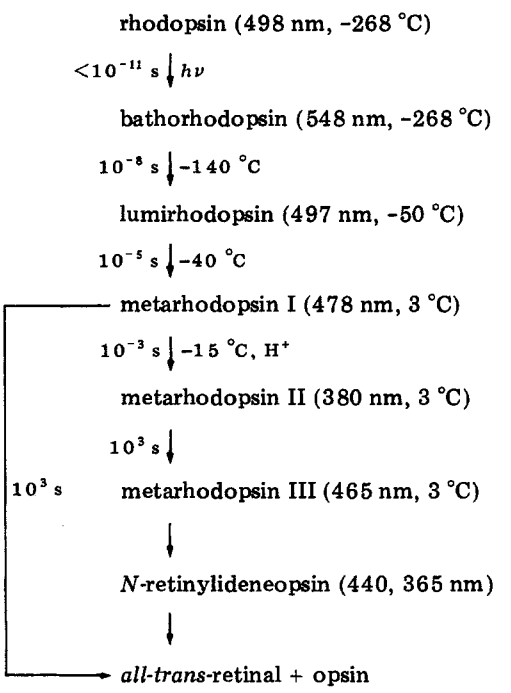
B. Rhodopsin Flash Photolysis

The initial act in vision is the absorption of a photon by the chromophore of a pigment molecule. This, in turn, causes a sequence of dark reactions in the rhodopsin molecule which can be readily monitored by means of the spectral changes accompanying them. Table II lists the rhodopsin photolytic intermediates, their absorption maxima, the temperature at which they are stable, and their lifetimes at room temperature.

Knowledge of the various intermediates was initially obtained from low-temperature studies in aqueous glycerol glasses. The first detectable photoproduct of micellar rhodopsin at liquid N_2 temperature is bathorhodopsin, absorbing at 540 nm.[233,234]

Warming bathorhodopsin in the dark to −140 °C or above causes conversion to the second photolytic intermediate, discovered by Broda and Goodeve (1941)[235] and later named lumirhodopsin by Wald. Further warming forms metarhodopsin I (MI) above −40 °C,[235] and above −15 °C this is in thermal equilibrium with a tautomeric form, metarhodopsin II[236,237] (MII). After MII the photolytic pathway appears to divide,[237-240] one

TABLE II. Bleaching Sequence of Vertebrate Rhodopsin[a]



[a] Listed are the names of the intermediates, their λ_{max}, the temperature at which they are stable, the temperature above which they undergo a thermal reaction to form the next intermediate, and the approximate rate of their conversions at room temperature.

fraction decaying directly to opsin and *all-trans*-retinal and the other fraction forming the intermediate, metarhodopsin III, (MIII), called "pararhodopsin" by Wald.[241] All the above intermediates, first discovered by means of low-temperature studies, have later been identified as genuine parts of the rhodopsin photolytic cycle at room temperature.[242-246] The same is not true in the case of a presumed sixth intermediate, absorbing at 430 nm and therefore called hypsorhodopsin.[247] Only Kobayashi, in a very recent picosecond flash photolytic study, finds a transient blue photoproduct preceding bathorhodopsin,[244] while all the other laboratories have provided evidence to the contrary.[242,243]

At the slow end of the time scale of rhodopsin photolysis, after MII, where the chromophore appears to be minimally perturbed by the protein moiety, there could exist additional intermediates, conformationally quite disinct from MII but with identical visible absorption spectra.[214,269] For example, the phosphorylated form of MII which is certainly different in its molecular structure from the unphosphorylated form has the same absorption spectrum. Furthermore, kinetic light-scattering data suggest that at least two additional rhodopsin conformational changes occur within a few hundred milliseconds after illumination, i.e., in a time span where there is no spectroscopic change other than the MI to MII conversion.[214,215]

In our discussion we shall focus on the rhodopsin to bathorhodopsin transition, the primary photochemical process in vision, as well as the early dark, thermal reactions in the photolysis cycle terminating at MII, since these processes occur within the time interval preceding receptor potential generation and, therefore, offer possible modes of coupling of rhodopsin to processes in the disk membrane. The decay of MII and the subsequent processes are far too slow to be involved

in transduction. These processes have been extensively reviewed by Baumann,[248] and by Ostroy[249] who has compiled almost all the data obtained so far on rhodopsin photolysis.

The Primary Photochemical Process

The initial photochemical process in vision involves the following steps: (a) the electronic excitation of the chromophore, (b) radiationless transitions in which the excitation energy is channeled into the particular state from where the initial chemical or physical event occurs, and (c) the initial event itself, in which the chromophore undergoes some intra- and/or intermolecular change with a concomitant transfer of a sizeable fraction of the electronic excitation energy to the protein environment. At the present time we do not have a clear picture of these processes. First, there is the question of the structure of the retinylidene chromophore; i.e., is the Schiff base nitrogen atom protonated, unprotonated, or hydrogen bonded in some fashion? The resonance Raman spectral data support the fully *protonated* model,[69-72] whereas ^{13}C NMR[74] and IR[73] spectral data on rhodopsin so far support an *unprotonated* or possibly *hydrogen-bonded* model. Obviously, this question is most germane to the possible pathways of the primary process.

If we assume that the chromophore is a fully protonated 11-*cis*-retinylidene group, then the logical pathway of the primary process is initial excitation to the lowest lying $^1\pi,\pi$ state populated by a fully dipole allowed transition. This may be followed by a radiationless internal conversion to a lower lying state having the same symmetry as the ground state. This state has apparently been seen in weak fluorescence emission.[250] While in this state or in the initially populated $^1\pi,\pi$, partial cis–trans isomerization occurs in times less than 10 ps to form bathorhodopsin. Now bathorhodopsin must exist in this highly strained form having an energy which is ca. 33 kcal/mol higher than that of the ground-state rhodopsin.[251] It is this quantity of energy which must be transferred in some way to the protein moiety of rhodopsin.

The mode of transfer of electronic excitation energy to the protein moiety has been recently dealt with by Lewis,[252] Warshel,[253] Honig,[254] and Cooper.[251] The problem is by no means simple as the chemical and physical pathways which we associate with such processes in small molecules appear to be precluded in the case of rhodopsin. Within the framework of the protonated Schiff base model, one likely mode for this transfer could be photoexcitation followed by partial twisting about the C_{11}–C_{12} double bond of the chromophore, to form bathorhodopsin. This would shift the positive charge from the Schiff base nitrogen atom the region involving the cyclohexane[255,256] ring. Such a massive shift in charge could transfer the energy by "breaking Coulombic bonds" existing in native rhodopsin, leading to the cascade of conformational changes occurring in the dark reactions of the photolytic cycle.

If we assume that the chromophore is not protonated but rather hydrogen bonded to some appropriate group,[68,257] then there is the possibility of *proton translocation* occurring which could effectively transfer the excitation energy to the protein moiety. This is illustrated in Figure 9. Peters et al.[242] have invoked such a transfer to explain their picosecond flash photolysis results in which they observe a large deuterium effect in the rate of the rhodopsin → bathorhodopsin conversion. Their proposed model, following that of van der Meer et al.,[257] involves proton transfer from the *cyclo*hexane ring of the chromophore to a "relay" group (presumably imidazole), which is initially hydrogen bonded to the Schiff base nitrogen. This results in a proton translocation from the imidazole to the fully protonated position on the Schiff base nitrogen atom.

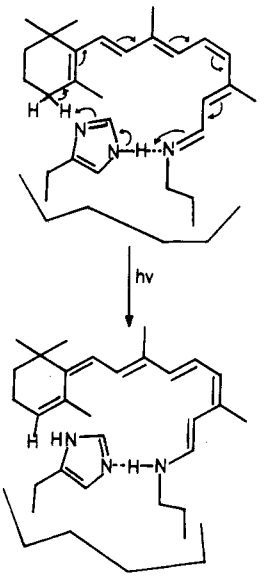


Figure 9. Hypothetical model for the primary process in vision, involving proton translocation.[243,257]

The principal difficulty with the proton translocation model, aside from the resonance Raman evidence favoring a fully protonated Schiff base in native rhodopsin, is the report that there is no difference in the N–H as well as N–D stretching frequency between rhodopsin and bathorhodopsin[258,259] when observed by Raman scattering. Moreover, it is very hard to reconcile how proton translocation could account for the high activation energy (ca. 36 kcal/mol) found for the thermal bleaching of rhodopsin.[254]

2. The Trigger Process of Transduction

It is logical to assume that one or more steps of the rhodopsin photolytic cycle prior to the decay of MII must be involved in the triggering of transduction. However, considering the various physical or chemical processes which come to mind as possible candidates, i.e., processes capable of communicating with the rhodopsin environment, one is led to the assumption that the trigger step does not occur prior to the decay of lumirhodopsin. Some possible modes of coupling of rhodopsin photolysis to transduction are rhodopsin on illumination, (a) forming an active pore, (b) binding or releasing a substance, (c) binding to some other membrane component, thus causing this component to form a pore or to become enzymatically active, or rhodopsin itself, being a light-stimulated enzyme. All the above listed processes require a light-dependent conformational change in the opsin, something which has not been observed prior to the lumirhodopsin decay.[102] Furthermore, in the case where the trigger action is not confined to the rhodopsin molecule alone, but requires

intermolecular interaction with other membrane components, the lifetime of the trigger form of rhodopsin obviously has to exceed the time required to establish this trigger action. Again, the first intermediate that meets this requirement is the lumirhodopsin → metarhodopsin I transition.[114-116] The first intermediate apparently to be involved chemically in *intermolecular* processes is the MI/MII transition, which is accompanied by a proton uptake[260-262] and which requires the presence of H_2O.[263] The other class of phenomena that could play a trigger role are electrical, i.e., charge displacements. Again, no such process is observed prior to the decay of lumirhodopsin: Cone and Cobbs report that the R_1 phase of the intercellular early receptor potential, whose amplitude is proportional to the extent of rhodopsin photolysis, seems to correlate roughly with the lumirhodopsin decay, the R_2 phase with the MI/MII transition.[264]

(a) *The Lumirhodopsin → Metarhodopsin I Transition.* Lumirhodopsin has a lifetime of ca. 3 μs at room temperature.[115,264] Unlike MI/II, it does not appear to involve its aqueous environment. Although there has been some question as to whether there is one or more than one parallel path for the decay of lumirhodopsin in bovine ROS, recent work appears to be consistent with two different parallel first-order processes.[265-267] This behavior has led to a model where two conformeric states of rhodopsin, which are in temperature- and pH-dependent equilibrium, are photolyzed via two parallel isochromic pathways with slightly different (by a factor of 4–6) rates.[265-267] In frog rhodopsin no such behavior could be observed.[268]

The lumirhodopsin decay is accompanied by a rapid electric event, the so-called R_1 phase of the early receptor potential ERP.[264] The molecular origin of this phenomenon seems to be a charge displacement within the opsin, which can also be measured in model membranes where rhodopsin is oriented on one side of a thin Teflon film.[269]

(b) *The Metarhodopsin I → Metarhodopsin II Transition.* The MI/II transition has been the earliest and most extensively studied of all reactions in the vertebrate photolytic cycle.[87,249] For micellar (digitonin) rhodopsin an equilibrium between MI and MII has been observed[236,237] which depends on the temperature, polarity, and pH of the medium, high temperature and polarity and low pH favoring MII. This equilibrium has also been observed in perfused frog retina[268] and isolated ROS[270,271] where the pH dependence of MII resembles a titration curve of a proton binding group with $pK \simeq 7$[126,266] (in digitonin the pK is ca. 6.3[236]). Ca^{2+}, when it has access to the disk lumen (interior), also affects the MI/II equilibrium, high (Ca^{2+}) favoring MI.[126,272]

The kinetics of the MI/II reaction show some unusual features. Early studies[273,274] of the kinetics of this reaction in digitonin micelles were consistent with the existence of *several forms* of MI each decaying to MII by a first-order process. However, early data taken on ROS[263,275] and in the excised eye (rabbit)[276] have generally been interpreted in terms of a single first-order process. More recent studies on ROS from two different laboratories favor *two* forms of MI, each decaying by first-order kinetics.[265-267] As this behavior extends also to "lipid free" rhodopsin,[265] it would seem that the origin of the two forms is intrinsic to the protein moiety, rhodopsin.

Further support for the concept that two conformeric states of rhodopsin, which are in pH- and temperature-dependent equilibrium, are photolyzed via two parallel pathways comes from the following: over a wide range of temperature and pH the fraction of lumirhodopsin, which decays via the fast pathway, is identical with the "rapid fraction" of MI.[265-267] Moreover, the two parallel pathways are also observed in the decay of MII, the slower pathway involving hydrolysis of MII directly to retinal and opsin and the faster one involving the intermediate MIII.[271]

The activation parameters[246,266] ($E_A \simeq 33$ kcal mol^{-1}) for the MI/MII process are consistent with the scission of 4–6 hydrogen bonds which usually accompany protein conformational changes, but CD spectral studies on rhodopsin in situ do not support this.[277] Alternatively, one might account for the activation parameters by assuming a change in either the electric charge on the protein and/or the amount of lipid or detergent tightly bound to it.[87]

In the majority of reports on MI/MII a linear Arrhenius plot is found for the apparent rate of MII formation.[246] For an equilibrium reaction, however, the apparent rate should be the sum of forward and reverse rate constants, $k_1 + k_2$. In a temperature range where k_1/k_2, i.e., the equilibrium constant, varies considerably with temperature (in bovine $k_1/k_2 = 0.5$ at 5 °C and $k_1/k_2 = 3$ at 25 °C), the Arrhenius plot should exhibit an increasing slope with increasing temperature.[126] The fact that this is not observed was explained by Hoffmann, who assumed a decreasing activation energy for MI/II with increasing temperature due to an altered lipid protein interaction.[266] As support for this suggestion he cites X-ray,[26,214] spin-label,[278] and fluorescence anisotropy[179,180] studies, all indicating a lipid-phase transition occurring over a considerable temperature range.

The extent to which lipids or a lipid-like environment can affect MI/II is apparent from a number of findings: in the detergent LDAO (lauryl dimethylamine oxide) the MI/II rate is increased 1000-fold[138] relative to ROS, whereas a lipid-free digitonin suspension of rhodopsin yields a 50-fold decreased rate.[265] In ROS membranes, but not in strong detergent solutions, high pressure shifts the equilibrium toward MI.[281] Since pressure can readily affect lipid-phase transitions, but very unlikely protein conformational changes, it is reasonable to speculate that MI/II might be coupled to a phospholipid chain order/disorder transition.

In ROS the MI/II reaction is accompanied by a concomitant uptake of one to two protons per bleached rhodopsin.[260-262,282-285] In the detergent Triton X-100, however, up to three protons are released and up to six protons are taken up, depending on the pH.[261] Like the total photolytic cycle, the proton uptake can be regenerated by addition of 11-*cis*-retinal.[282] It also can be photoreversed; i.e., near-UV illumination of MII (380 nm) causes proton release.[283]

Although occurring on a similar time scale, proton uptake and MI/II reaction do not seem to be closely linked. Neither do the kinetics of the two processes completely match (D. Emeis, personal communication), nor is the proton uptake, like MI/II, an equilibrium

process, since its extent is unchanged at low temperature, where the MI/II equilibrium lies on the side of MI.[262,284,285]

There is a light-induced birefringence change in the frog retina (Liebman et al.)[227] which appears to reflect a reversible transition from one membrane state associated with MI to another one associated with MII. The same is true for a rapid, light-induced refractive index change N in the near-infrared which supposedly arises from the change in the anomalous dispersion (Cotton effect) spectrum accompanying the spectral transition from MI to MII[126,165] and for the R_2 phase of the ERP.[264]

If one assumes that the MI/MII transition, described by reaction 1,

$$\mathrm{MI} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{MII} \qquad (1)$$

or accompanying equilibrium processes is in some way coupled with membrane events so as to trigger transduction, one has the problem that in visual process the bleaching of *one* rhodopsin molecule should lead to *one* chain of events terminating in a *single* act of transient hyperpolarization of the plasma membrane. Now the observed rate constant of the MI/II reaction is, under physiological conditions, ca. 1000 s^{-1}, while the equilibrium constant is about 10. This implies that each MI molecule proceeds to MII and returns with a rate of about 90 times per second before the eventual decay of MII to MIII or retinal + opsin. It is difficult to imagine how such a shuttle process can trigger visual transduction unless there is some unique character of feature or the initial shuttle cycle.

One of us, R.U., has recently suggested a scheme for the MI/II reaction which contains an initial irreversible step which provides the needed feature.[214] The scheme was proposed on the basis of a kinetic comparison of the molecular MI/II transition and a rapid light scattering transient signal, "P", reflecting a light-induced shrinkage of the disk organelle.[216,217] This disk shrinkage relates kinetically to MI/II, i.e., its activation parameters and pH behavior are similar but its extent does not depend on the equilibrium between MI and MII. Equation 2 given below embodies the required initial irreversible step (k_1). The equilibrium is established between MII and an isochromic form of MI, MI', which lacks the coupling feature of MI:

$$\mathrm{MI} \xrightarrow{k_1} \mathrm{MII} \underset{k_3}{\overset{k_2}{\rightleftharpoons}} \mathrm{MI}' \qquad (2)$$

Such a scheme could explain many of the discrepancies found in the literature regarding the MI/II transition.[126,217,237,266,267]

Further support for the scheme of eq 2 comes from the measurement of the kinetics of MII formation in the equilibrium, stimulated by a T-jump pulse. The kinetics are found to be clearly different from the MII formation in the course of rhodopsin photolysis (H. Rüppel, personal communication). It therefore appears possible that the irreversible decay of MI triggers transduction and that the so-called MI/II equilibrium—here the MII/MI' equilibrium—is a postponed reaction.[217]

C. Light-Induced Enzymatic Activities

1. Phosphodiesterase Activity

The fastest light-induced enzymatic activity measured so far in the ROS is the onset of a cyclic guanosyl monophosphate (cGMP) specific phosphodiesterase (PDE).[286-289] ROS have an unusually high cGMP content of ca. 10–30 μM in the dark.[290] On illumination this drops about 50–70%, and the cGMP concentration remains low until illumination ceases.[208,210] By means of rapid acid quenching techniques, Woodruff et al.[210,291] could show that bleaching of one rhodopsin can lead to the hydrolysis of 5×10^4 molecules of cGMP, with a half-time of 125 ms. This and the short latency of the enzymatic light response (less than 50 ms) would be consistent with the involvement of cGMP hydrolysis in the mechanism of visual transduction.[125,210,291]

The decline in cGMP concentration becomes larger as the illumination intensity increases. Half-saturation of the response is obtained when about 200–1000 rhodopsin molecules are photobleached, i.e., the "light sensitivity" of the enzyme is 1–2 orders of magnitude lower than that of the photoreceptor potential of the dark adapted retina.[2,4,167,168,210] Liebman reports that both in frog and in bovine ROS the bleaching of a single rhodopsin is sufficient for the eventual activation of all PDE molecules on one disk.[292,293] He estimates that there are between 29 and 500 rhodopsin molecules per single PDE molecule, which implies a primary amplification step with a gain of between 29 and 500. Together with the second amplification, the turnover rate of single PDE molecules, this yields a total amplification of 40 000–50 000 (cGMP molecules hydrolyzed per second per rhodopsin molecule bleached).[125,210,291,293] More massive flashes of light, bleaching more than one rhodopsin per disk, also turn on all PDE molecules but with a greatly reduced lag phase.[291-293]

Phosphodiesterase activation requires not only light but also the presence of small amounts of GTP, i.e., 0.06–0.5 μM of GTP are sufficient to cause half-maximum activation.[167,168,292,293] When ROS are illuminated in the absence of GTP, only a very low phosphodiesterase activity is observed, which drastically increases upon addition of GTP.[292] In the native system GTP is hydrolyzed,[167,168,292,293] but it can be replaced as cofactor by GTP analogues which are not hydrolyzable (methylene GTP or GMP-PNP).

The ROS phosphodiesterase is loosely bound to the disk membrane and can be reversibly washed off in Mg-free solutions.[167,168,287,289] In bovine photoreceptors the solubilized enzyme is monomeric (M_r 125 000) and is active even in the dark.[294] In the frog ROS the isolated enzyme is a dimer (M_r 240 000) and is inactive but can be activated by proteolysis.[168,287] The inactivation of the enzyme in the dark-adapted disk membrane appears to be regulated by an inhibitor protein (M_r 26 000).[295]

Liebman[293] has suggested a model where photobleached rhodopsin molecules (probably MII) randomly diffuse in the disk membrane until they collide with, and bind to, one of the 29–500 phosphodiesterase–inhibitor complexes, thus activating the enzyme. GTP in this model binds to the MII–inhibitor complex, releasing the MII for further activation of other phosphodiesterases in the same disk. In this way one single

bleached rhodopsin can gradually turn on all the phosphodiesterases in one disk, provided enough GTP is present.

Assuming that the light-dependent phosphodiesterase activity is involved in the transduction mechanism, the Liebman model would predict that the quantity of light seen by the photoreceptor cell is no longer decoded as a simple analogue value like amplitude or an "activity", but rather as a time-integration phenomenon; i.e., since all PDE molecules will eventually be activated, independently of the number of bleached rhodopsins per disk, the final PDE activity will no longer contain the original light-intensity information. However, since low photobleaches require a much longer time for all the enzymes to become activated, the light-intensity information will still be contained in the amount of cGMP hydrolyzed within a given time interval. This interval could be the time between the rhodopsin bleaching and some reaction, probably also light dependent, that turns the PDE off. Alternatively, the capability of MII to activate the enzyme could decay in a rapid dark reaction of the pigment molecule which is not accompanied by spectral changes.

An alternative model for the phosphodiesterase activation was proposed by Bitensky and co-workers. It will be described at the end of the next section, since it involves the rapid light-induced GTPase activity described there.

2. Light-Activated GTPase

The concentration of nucleotide triphosphates in frog ROS is extraordinarily high, amounting to a few mM for ATP and GTP.[159,166,296] Upon illumination the ATP concentration rapidly drops in consequence of a rapid, light-induced GTP hydrolysis, followed by phosphate transfer from ATP to GDP.[166] The GTPase responsible for this process has the same light sensitivity as the phosphodiesterase discussed above, i.e., 1 rhodopsin bleached in 200 gives half-maximum activation.[167,168]

The activation of the enzyme appears to be an irreversible process since the activation curve (activity vs. log light intensity) is exponential rather than hyperbolic (Robinson, personal communication). The K_M of the GTPase is around 0.6 μM, very close to the GTP concentration required for half-maximum activation of the phosphodiesterase. The maximum GTP hydrolysis rate was found to be 5 molecules of phosphate per second per rhodopsin molecule bleached.

Like the phosphodiesterase the GTPase can be easily washed off the disk membrane in a Mg^{2+}-free Ringer solution,[166-168] but its activity is readily restored by recombining pellet and supernatant of this washing procedure in a Mg^{2+}-containing solution.

The GTPase activity, according to Robinson and Hagins, functions by providing metabolic energy to the light-exposed receptor cell so as to restore the dark-adapted state.[166] Bitensky's group, which later reported on the same GTPase system, on the other hand, suggested that the GTPase plays a crucial role in regulating the GTP-dependent phosphodiesterase activity[167,168] which constitutes an alternative model to that proposed by Liebman for PDE activation.[293] They argue that not only light but also a small fraction of bleached disks mixed with a dark-adapted disk preparation can activate the GTPase. This occurs even when the bleached disks are depleted of their phosphodiesterase. On the other hand, the phosphodiesterase activation of dark-adapted disks by bleached disks requires the presence of the GTPase. From this and from the finding that the same GTP concentration is required in order to half-maximally activate both PDE and GTPase, Bitensky concludes that it is the active form of the GTPase which, in turn, activates the PDE.[297]

3. Rhodopsin Phosphorylation

Light-stimulated phosphorylation of rhodopsin, first described by Kühn et al.[298] (bovine) and Bownds[299] (frog), is the best studied of the light-activated enzyme reactions in ROS. There is a rhodopsin-specific kinase in ROS, which appears to be loosely bound to the disk membrane in the dark[300,301] but strongly bound upon illumination.[301] Its molecular weight has been estimated at 53 000[302] and 67 000.[301] Photobleached rhodopsin is apparently its only known substrate,[303,304] and it is the γ-phosphate group of either ATP or GTP[303,304] that is transferred to the serine and threonine groups of rhodopsin with a half-time of 1 min at 37 °C.[305] A maximum of eight phosphate groups incorporated per bleached rhodopsin molecule has been reported.[306,307]

In dark-adapted frog retinas it appears as though low photobleaches (less than 10% rhodopsin photolyzed) cause not only the phosphorylation of the bleached rhodopsins but also phosphorylation of unbleached molecules.[308] Similarly rhodopsin, once bleached, is recognized as a substrate of the kinase, even after it has been regenerated with 11-*cis*-retinal.[309,310]

According to Shichi, only rhodopsin molecules in the plasma membrane and in the freshly formed disks at the basal end of the outer segment are phosphorylated.[302] Autoradiographic studies of Paulson et al., however, contradict this, showing an absolutely homogeneous ^{32}P incorporation into the intact ROS.[307]

Finally, we should remark that rhodopsin phosphorylation is too slow to be part of the visual transduction chain. It may, however, play an important role in adaptation processes.[305,310]

4. Other Light-Dependent Enzyme Activities

Thacher[164] recently reported the existence of a Mg^{2+}-dependent ATPase in frog ROS, the activity of which is increased 2-fold upon illumination. Its role in the outer segment has not yet been established. The Mg^{2+} ATPase recently described by Uhl et al.,[165,171,218] though *not* light stimulated, is also connected with light-dependent processes in the disk membrane. Its dark activity somehow "energizes" the disk membrane such that upon flash illumination a rapid (ms range) light-scattering transient can be observed which does not occur without ATPase activity prior to illumination.

Of the three low molecular weight proteins which Bownds' group reported to be phosphorylated in the dark, two are dephosphorylated upon illumination.[125] Photolysis of 1 in 10 000 rhodopsin molecules causes the dephosphorylation of 30% of the proteins.[125] One possibility is that the protein components might be associated with the "Na^+ pore" which, in the phosphorylated state, keep the pore open; dephosphorylation would close it.[125] In bovine and rat ROS a similar phenomenon is observed, the only difference being that the protein phosphorylated in the dark (in a cGMP-

dependent manner) and dephosphorylated in the light has a molecular weight of 31 000.[172]

D. Light-Induced Structural Changes in ROS

1. Changes Observed by Light Scattering

There have been a number of reports dealing with rapid, light-induced scattering changes within ROS.[126,165,171,215-226]

Aside from the light scattering transient connected with the Mg^{2+}ATPase mentioned above, two classes of light-scattering intensity increments appear to be of particular significance: a rapid, light-induced disk shrinkage first studied in bovine ROS[126,215-217,220,225] and later in the frog ROS.[224] For photolytic bleaches up to ca. 3%, the extent of the observed disk shrinkage increases linearly with the amount of rhodopsin bleached per flash. For more massive bleaches, however, the response amplitude fails to increase proportionally and saturation sets in at about 10% bleaching.[126,215,216,225] This would indicate that some sort of adaptive behavior is associated with the observed disk shrinkage. The molecular basis of the disk contraction is, as yet, not known, but studies so far indicate that it does not arise from ion fluxes across the disk membrane.[215,224]

In the intact frog retina, stimulated by physiological bleaches, another light-scattering transient is observed.[226] Again, the molecular processes responsible for the underlying structural changes is not yet known. It is important, however, to keep this and the other light-scattering transients in mind as possible artifacts when attempting to measure light-induced ion fluxes by optical (absorptive) means, using indicator dyes.

2. Changes Observed by Birefringence

The rapid, flash-induced birefringence increment in the frog retina[227] which appears temporarily coupled to the MI/II transition could logically arise from a change in the lipid order parameter accompanying charge and/or conformation changes in the pigment molecule.

3. X-ray Diffraction

A number of structural changes in ROS upon illumination have been observed by Chabre and Cavaggioni,[214] using X-ray diffraction and a rapid detection system, which reduces X-ray exposure times to about 30 s. One response they found, namely, a reduction in the disk–disk lattice distances of ca. 1% that appeared to saturate at about 10% bleaching, could be associated with disk shrinkage monitored by light-scattering techniques.

VII. Transduction Models

In the processes which communicate the photosignal from light-activated pigment molecules to the plasma membrane, a variable amplification step with a gain of between 10^7 and 10^2 is involved.[2,4] A gain of 10^7 means that a single bleached rhodopsin effectively suppresses the influx of ca. 10^7 Na^+ into the outer segment.[51,153,158,159] When the receptor cell works at its highest sensitivity, i.e., in the dark-adapted state, the quantum efficiency of visual perception is 0.6, essentially the value found for the quantum efficiency of photoisomerization of the pigment molecule rhodopsin.[3,311] This implies that once a rhodopsin molecule is photobleached, the probability that this leads to a receptor potential well above the thermal noise level of the receptor cell is approaching unity.

It is now generally assumed that the communication process between the rhodopsin-containing disk membrane and the ROS plasma membrane involves a diffusable transmitter.[312] Such a model could explain how the photolysis of a single rhodopsin in the disk membrane, i.e., a change on *one* photopigment molecule in 10^7–10^9 in a single rod, causes the transient blockage of at least 10 Na^+ channels in the plasma membrane (thus preventing ca. 10^7 Na^+ from entering the cell) without there being a morphological or electrical connection between the disk and plasma membranes.[313,314] Furthermore, the complex sequence beginning with the activation of a change in transmitter concentration and terminating with the restoration of the original transmitter concentration could account for the complex kinetics of the rise of the receptor potential, which has been described in terms of 4–10 consecutive first-order processes.[151,315]

The diffusable transmitter is invoked to occupy specific binding sites in the plasma membrane which, in turn, regulate the permeability of Na^+ channels. Two mechanisms come to mind that could account for the light regulation of these ion channels: (1) When a transmitter binds to the channel, the channel is closed; therefore light would have to increase the cytoplasmic concentration of the transmitter by releasing or generating it. (2) The pore is closed in the absence of transmitter; i.e., light would have to affects its removal or destruction.

In what follows we shall demonstrate how even such a simple transmitter model might explain many of the features of the input–output relation of the receptor cell.

A. Simple Transmitter Model of Transduction

The light response curve of a single photoreceptor cell as shown in Figure 2 is best described by the equation of Naka and Rushton[316]

$$\frac{V}{V_{max}} = \frac{I}{I + \sigma} \tag{3}$$

where V is the amplitude of a receptor potential caused by a short flash of light of intensity I and V_{max} is the maximum response amplitude; σ is a measure of the sensitivity of the receptor cell and is defined as the light intensity required for a half-maximal response, $V_{max}/2$.

When it is assumed that the binding and dissociation at the transmitter binding sites are sufficiently fast to permit at least near-equilibrium reaction at the peak of the photoreceptor response, a pore-transmitter dissociation constant, K, can be given, relating free transmitter concentration, [T], in the cytoplasm, the "pore concentration", [P], and transmitter bound to the pore, [TP]:

$$[T][P]/[TP] = K \tag{4}$$

[Pore concentration can be visualized as the number of pores in the outer segment plasma membrane divided by its total cytoplasmic volume.] Now letting α_1 be the fraction of open pores at a given transmitter concentration $[T_1]$ and α_2 the fraction of open pores after light of intensity I has increased the cytoplasmic transmitter

concentration by a proportional amount xI, the fraction of pores closed by light relative to the fraction of pores open prior to illumination is given by eq 5, which can

$$\frac{\alpha_1 - \alpha_2}{\alpha_1} = \frac{\frac{K}{K + [T_1]} - \frac{K}{K + [T_1] + xI}}{\frac{K}{K + [T_1]}} \quad (5)$$

be arranged to give

$$\frac{\alpha_1 - \alpha_2}{\alpha_1} = \frac{I}{I + \frac{K + [T_1]}{x}} \quad (6)$$

Clearly, $(\alpha_1 - \alpha_2)/\alpha_1$ equals V/V_{max}. Therefore, the response characteristics of a receptor cell can be adequately described by a simple diffusable transmitter model provided that there is a linear relationship between light intensity and the increase in free transmitter concentration in the cytoplasm. This condition is met as long as the "pore concentration" is considerably less than the pore-transmitter dissociation constant K.

Comparison of eq 3 and 6 reveals that the inflection point $\sigma_{(T_1)}$ of the sigmoidal response curve shifts toward higher light intensities when (T_1), the cytoplasmic transmitter concentration in the dark-adapted state, increases. Thus

$$\sigma_{(T_1)} = \sigma_0 \frac{K + [T_1]}{K} \quad (7)$$

where σ_0 is the light intensity required for half-maximum response at a transmitter concentration $[T] = 0$.

At the same time the concentration of pores available for blockage decreases with increasing $[T_1]$ is

$$[P] = [P_0] \frac{K}{K + [T_1]} \quad (8)$$

when $[P_0]$ is the total pore concentration. Thus the dynamic range of photoreceptor cell, as measured by [P], is compressed by the same factor as the sensitivity, $1/\sigma$, is decreased.

In terms of a single transmitter model, a steady background illumination should give rise to an elevated cytoplasmic transmitter concentration which would account for part of the observed background adaptation. However, since the observed increase in σ in vivo far exceeds the observed compression of the dynamic range (see Figure 3), additional mechanisms must be involved in background adaptation. For example, if x, i.e., the increase in transmitter concentration produced per unit increment of light intensity, were to decrease with increasing background light levels, the simple transmitter model described by expression 4 could account not only for the high gain of the transduction process but also for the mechanism that automatically controls this gain in accommodating to very different light levels.

All the above arguments also apply to an "antitransmitter" system, i.e., a system where light causes the uptake or destruction of a transmitter substance which binds to the Na^+ pores and keeps them open.

B. Ca^{2+} Hypothesis of Visual Transduction

Of the various candidates for the role of transmitter, Ca^{2+} is the one that has caught the most attention over the past decade. It was first introduced as a possible transmitter substance by Yoshikami and Hagins in 1971;[317] they suggested that the disks in the outer segment serve to store Ca^{2+} in the dark and to release it upon illumination for diffusion to the plasma membrane where it closes Na^+ channels. The evidence supporting this model is the following:

(1) Externally applied Ca^{2+} rapidly (<1 s) and reversibly suppresses the dark current in rods by reducing the Na^+ permeability of the plasma membrane,[51,153] thus mimicking the action of light. Since in most neuronal membranes Ca^{2+} exchanges rapidly across the plasma membrane, the external effect of Ca^{2+} on the photoreceptor is assumed to be an internal one.[314]

(2) In the presence of the Ca^{2+} ionophores X537X or A23187 the rods are strongly sensitized to external Ca^{2+};[314] i.e., the external Ca^{2+} required to suppress 95% of the dark current is reduced from 20 mM to about 10 μM. Ca^{2+} therefore seems to work in the cytoplasm at μM concentrations, mimicking the action of light.[318,319]

(3) Exposing rods to low Ca^{2+} concentrations immediately depolarizes the cell, expanding the dynamic range accordingly.[153,201,314,319] In rat rods, dark current and the maximum response in low Ca^{2+} (pCa > 5) are up to 5-fold higher than in normal Ca^{2+} (pCa $\simeq$ 3). In *Bufo marinus* the increase is only 2-fold.[201] In both animals, however, short exposure to low Ca^{2+} only expands the dynamic range of the response without shifting the sensitivity.[319,201] This short time effect of low Ca^{2+} is viewed as a cytoplasmic one due to reduced blockage of Na^+ pores.[319,201] In terms of the Ca^{2+} hypothesis, this would indicate that in the dark adapted rat rod, 80%, and in the frog rod, ca. 50% of the Na^+ pores are already blocked.

Prolonged exposure of rods to low Ca^{2+} (pCa $\simeq$ 9) gradually desensitizes the cell. Hagins reports that in low Ca^{2+} the light intensity required for half-maximal response increases from 30 to 300 photons per rod and stays there for several hours,[314,319] whereas Lipton et al.[201] find that the sensitivity of *Bufo marinus* retinas gradually decreases until, after 12 min, no response can be obtained. In terms of the Ca^{2+} hypothesis these data were interpreted as depletion of the Ca^{2+} stores in the disk.

(4) Ca^{2+} chelators, introduced into rat rods at 10 μM concentrations by the vesicle fusion technique, reduce the size of the cell response to weak light flashes (<100 photons per rod per flash) but not to bright flashes,[207,313,318] suggesting that the transmitter molecule is either Ca^{2+} or a small organic molecule with an affinity for the Ca^{2+} chelators equal to that of Ca^{2+} itself.[318]

(5) Supposedly the strongest evidence for the Ca^{2+} hypothesis comes from measurements at very low Ca^{2+} ($<10^{-9}$ M) on cones, where the intradiscal space coincides with the extracellular one. The response of the cell vanishes, but returns when the original Ca^{2+} level is restored.[318]

C. Evidence against the Ca^{2+} Hypothesis

(1) Various estimates have been made as to the number of Ca^{2+} ions the disks would have to release per single bleached rhodopsin molecule in order to account for the observed light response, i.e., for the fact that in the fully dark adapted receptor cell one photon leads

to the blockage of 3% of the available Na^+ channels and 30 photons yield half-maximum response.[153,314,320] These estimates range from 20 to 1000 Ca^{2+} released per bleached rhodopsin. However, a very simple calculation which we here submit indicates that considerably higher numbers are required.

Provided that the Na^+ pore binding site for Ca^{2+} is the only Ca^{2+} buffer in the cytoplasm, and since we know α_1, the fraction of open pores in the dark-adapted photoreceptor cell, and σ, the number of photons required for half-maximum response, eq 6 allows the calculation of x, the transmitter-concentration increase in the cytoplasm per bleached rhodopsin molecule:

$$x = \frac{K + [T_1]}{\sigma} \tag{9}$$

σ = 30 photons,[313,314,318,319] α_1 = 0.2 in rat rods[153] and 0.5 in frog rods,[201] and therefore $[T_1]$ can be calculated from (10):

$$[T_1] = \frac{(1 - \alpha_1)K}{\alpha_1} \tag{10}$$

Taking K to be 1 μM,[318] $[T_1]$ becomes 4 μM in the rat rod and 1 μM in the frog. Therefore one photon would have to increase the cytoplasmic Ca^{2+} concentration by 1.66×10^{-7} M and 6.66×10^{-8} M in rat and frog rods, respectively. Taking the aqueous volume of the cytoplasm of the rat ROS to be 2.7×10^{-14} L,[320] this would mean that a release of about 2700 Ca^{2+} per bleached rhodopsin is required. In the frog rod, with its much bigger volume (7×10^{-13} L[305]), the released Ca^{2+} would be "diluted" even further, making the number for the required Ca^{2+} release about 28 000. Moreover, in the presence of a Ca^{2+} buffer in the cytoplasm these numbers would become even higher:

For a Ca^{2+} buffer in the cytoplasm with a dissociation constant C, a total concentration of $[B_0]$, and a free concentration of [B], the concentration of Ca^{2+} bound to this buffer, i.e., [CaB], can be derived from eq 11.

$$\frac{[Ca][B]}{[CaB]} = C \tag{11}$$

Since $[B] = [B_0] - [CaB]$, it follows that

$$[CaB] = \frac{[Ca][B_0]}{[Ca] + C} \tag{12}$$

The ratio Y of buffer-bound to free Ca^{2+} is therefore

$$Y = \frac{[CaB]}{[Ca]} = \frac{[B_0]}{[Ca] + C} \tag{13}$$

If we were again to neglect the very small amount of Ca^{2+} bound to the pores in the plasma membrane, then in the presence of a Ca^{2+} buffer, $(Y + 1)$ times as many Ca^{2+} must be released per bleached rhodopsin compared to the unbuffered case. Equations 10 and 13 together yield

$$Y = \frac{B_0}{\frac{1 - \alpha_1}{\alpha_1} K + C} \tag{14}$$

which, for the rat rod, becomes $Y_r = B_0/(4K + C)$, and for the frog rod, $Y_f = B_0/(K + C)$.

There are a number of Ca^{2+} buffers present in the cytoplasm, for example, about 3–5 mM of nucleotide triphosphates[296] with a Ca dissociation constant of the Ca–NTP^{2-} complex of 5×10^{-5} M. Thus, if all ATP and GTP were present in the form NTP^{4-} and none of it were complexed by any other ion but Ca^{2+}, Y_r and Y_f would become, respectively, 55–90 and 60–100. Even if, due to H^+ and Mg^{2+} binding, the concentration of free NTP^{4-} were as low as 10^{-4} M, reducing Y_r and Y_f to about 2–3, still 3 or 4 times as many Ca^{2+} would have to be released in the presence of the nucleotides in order to obtain a given response. In rat rods this raises the figures to ca. 8000–11 000 and in frog rods to ca. 80 000–110 000!

Additional Ca^{2+} binding groups present in the outer segment are the phospholipids, which have a relatively small binding constant but are present in very high concentrations, and the mucopolysaccharides. The proposed Ca^{2+} release figures therefore appear to be lower limits.

The above calculations, admittedly, are based on several simplifications. They appear to indicate, however, that the number of Ca^{2+} a single bleached rhodopsin has to release in order to explain the observed light response is considerably higher than was previously assumed.

(2) So far, every attempt to demonstrate a light-induced Ca^{2+} release from ROS disks, which is large and fast enough to meet the requirements of the Ca hypothesis, has failed. These attempts include careful studies of rod cells under near physiological conditions[318,321,322] and studies of partially destroyed receptor cells, i.e., disks,[322-324] sonicated disk vesicles,[169,325,326,327] and lipid vesicles in which rhodopsin was incorporated.[125,324,328] Either no Ca^{2+} release is measured or one observes the formation of a Ca^{2+} pore the transport rate of which is much too slow to account for the postulated release rate or a one-shot Ca^{2+} release (again with much too low a rate). To prove the Ca^{2+} hypothesis it will be necessary to show not only that Ca^{2+} mimics the action of light but also that the effect of light is to actually provide this Ca^{2+} in the cytoplasm, in the required time.

(3) Ca^{2+} release data of Kaupp et al.[322] from intact bovine ROS, in the presence of the Ca^{2+} ionophore A23187, appear to indicate that Ca^{2+} diffusion from the disk to the plasma membrane takes as long as 300 ms. (In frog ROS, which have about 6 times larger diameter, this diffusion could take even longer.) Compared to the 1-ms computed value for H_2O, this more than 100-fold retardation can be rationalized in terms of the high density of negatively charged Ca^{2+} binding sites in the cytoplasm (NTP^{4-}, phospholipids, mucopolysaccharides) and the greatly increased viscosity of the gellike matrix between the disks.[34,48] If Ca^{2+} were the transmitter molecule, one would expect its diffusion time from the bleached rhodopsin site to the plasma membrane to be one of the rate-limiting steps in the rise of the receptor potential. Single quantum bumps in the photocurrent should, therefore, show a considerable variability in their rise time depending upon whether the photolyzed rhodopsin was close to the plasma membrane or not. This, however, has never been observed.[329,330]

(4) There is another observation which seems inconsistent with the Ca^{2+} hypothesis. As already mentioned above, it has been reported that the immediate action of an extracellular Ca^{2+} concentration change is a change in membrane potential and V_{max}, but not σ.[201,319] If this effect were due to an immediate change in cytoplasmic Ca^{2+}, resulting in a blockage or opening of Na^+ pores, eq 7 would predict that the sensitivity is affected as well, since σ varies with $[T_1]$. In the case of the rat rod, where the dark-adapted value of $\alpha_1 = 0.2$, i.e., $[T_1] = 4K$, removing most of the cytoplasmic Ca^{2+} should not only increase V_{max} 5-fold but also decrease σ 5-fold, i.e., from 30 photons per rod to 6 photons per rod for a half-maximal response. This is not observed.

(5) Yoshikami and Hagins[153] report that prolonged exposure of rat rods to low Ca^{2+} (10^{-9} M) desensitizes the rods considerably. According to Lipton et al.,[201] rods even become completely desensitized after 10 min in EDTA, presumably due to Ca^{2+} depletion of the disks. However, Schnetkamp[175] reports that disks, both in intact and in leaky rods, lose their Ca^{2+} content only very slowly ($t > 2$ h) when exposed to an EGTA-containing medium.

On the other hand, in the presence of Ca^{2+} ionophores (X537A, A23187), at an external Ca^{2+} concentration of 10^{-7} M, no desensitization is observed, even after long incubations.[159,314] This, again, is in conflict with the result of Schnetkamp:[175] Due to the fact that in a very short time the ionophore is equally distributed in both the plasma and disk membrane, in low Ca^{2+} the disks are very rapidly depleted of Ca^{2+} in the presence of the ionophore. If Ca^{2+} were the transmitter and if it were stored inside the disk in the dark, addition of a Ca^{2+} ionophore should completely desensitize the cell.

(6) The light-induced influx of Ca^{2+} ions in cone cells is suggested to arise directly from the extracellular space. Therefore, the finding that lowering the extracellular Ca^{2+} levels below 10^{-8} M abolishes the light response of the cell has been taken as evidence for the transmitter role of Ca^{2+}.[318] However, as Bertrand et al.[331] as well as Arden and Low[332] report, the receptor potential of Ca^{2+} depleted cones does not vanish immediately, but with a time course very similar to that found in rod cells, where the proposed Ca^{2+} storage site is shielded from the extracellular space. It is very difficult to reconcile this finding with the Ca^{2+} hypothesis in its original form.

(7) Perhaps the strongest argument against the Ca^{2+} hypothesis is based on studies by Szuts.[333] Physiologically intact retinas were perfused in a medium containing the pure isotope $^{45}Ca^{2+}$ and exposed to a repetitive sequence of 1 min of light and 1 min of dark periods for 1 h. If light-stimulated Ca^{2+} release and subsequent reuptake were part of visual transduction, after 1 h the disks should contain almost exclusively radioactive Ca^{2+}. It was found, however, that only ca. 10% of the Ca^{2+} content of the disks was due to $^{45}Ca^{2+}$, making it very unlikely that Ca^{2+} release and uptake are significant properties of ROS disk membranes.

D. Cyclic GMP Hypothesis

Recently Lipton et al.[201,202] have demonstrated that Ca^{2+} is not unique in mimicking the action of light. Cyclic nucleotides, e.g., cGMP, appear to have very similar effects on the properties of the photoreceptor light response. Perturbations of the living system which are thought to increase the levels of cGMP in the outer segment cause effects comparable to the ones of decreased Ca^{2+} levels, and lowering the cytoplasmic cGMP appears to match the effects of increased Ca^{2+} levels.[334] These findings have been confirmed by Miller and Nichol[335,336] and by Waloga and Brown.[337] It therefore appears possible that cGMP could play the role of a ("negative") transmitter in visual transduction, i.e., in the presence of cGMP Na^+ pores would remain open, which would otherwise be shut.[293]

In contrast to the Ca^{2+} hypothesis, the cGMP model would have the advantage that it has been shown that ROS do contain a light-dependent mechanism that can effect a change in transmitter concentration which is large enough and probably sufficiently rapid (see section VIC). The effects which Ca^{2+} has on the receptor potential could then be understood in terms of a model where cGMP and Ca^{2+} are interrelated messengers (cGMP being the primary transmitter), controlling each other's concentration. For example, as Cohen demonstrated recently,[208] Ca^{2+} considerably affects cyclase activity in the retina, thus regulating cGMP levels in the outer segment. Several models of interrelated Ca^{2+}cGMP transmitter systems are discussed in detail by Lipton et al.[202] and Cohen et al.,[208] and some specific suggestions as to the mechanism of cGMP-dependent permeability regulations have recently been discussed by Liebman and Pugh[293] and by Hubbell and Bownds.[125]

There is, however, some very strong evidence against cGMP as the primary transmitter. Recent studies on the living perfused retina indicate that there, in contrast to isolated ROS, light-induced cGMP hydrolysis is slow, requiring several minutes to reach substantially decreased cGMP levels.[209] This would point to cGMP, as in many other systems studied so far, as playing an important role in regulatory processes (adaption) in the receptor cell rather than being the primary messenger. [*Note added in proof*: In this context it is interesting to note that the disk membrane Mg-ATPase described in V,B is not only active in the dark, but also transiently activated upon illumination and that this light-induced activity is regulated by cGMP concentrations in the physiological range (T. Borys, R. Uhl, E. W. Abrahamson, in preparation).] The much faster rate of hydrolysis in ROS might be explained by the fact that the pH optimum for PDE activity is 8,[210,292,293] and it was at this pH that the ROS experiments were run (since isolated ROS plasma membranes are permeable to both H^+ and Cl^-,[158] intra- and extracellular pH can be assumed identical). In the intact retina, however, there appear to exist pH gradients across the plasma membrane, and the intracellular pH was determined by Hagins and Yoshikami to lie around 6.8.[313,318]

E. Outlook

At present most hypothetical models for the mechanism of visual transduction incorporate a diffusable transmitter that mediates between rhodopsin and the plasma membrane. However, such models raise further questions which have yet to be answered satisfactorily:

(1) From the invariability of the time course of single quantum bumps[329,330] we know that the diffusion of any possible transmitter molecule species cannot be a

rate-limiting step in the rise of the receptor potential and must therefore be assumed to be relatively fast. On the other hand, however, rod cells can be illuminated locally such that the photoresponse from this part of the cell is saturated for minutes, indicating that locally there must exist an excess of transmitter molecules far beyond the level required for the blockage of all the sodium pores in this area. The same photoreceptor cell can still behave as if dark adapted when test flashes are subsequently applied to other previously unilluminated areas.[53,338,339] It is very difficult to imagine a mechanism that prevents high local transmitter concentrations in the cytoplasm from spreading along the long axis of the photoreceptor. But even if such a mechanism existed, in the case of cGMP as transmitter, another difficulty would arise: the enzyme which is thought to restore the original cGMP levels is a guanosine nucleotide specific cyclase presumably located in the cilium, which extends only a few micrometers into the outer segment.[173] This, again, is in conflict with the existence of the local illumination effect described above and the invariability of the kinetics of single quantum bumps.

(2) The cytoplasmic space, whose volume varies greatly from species to species (see Table I and the calculations in section VIIC), appears to be a poor candidate for the "reaction vessel" in which a concentration-regulated primary transduction step takes place. This is because the machinery responsible for the production and subsequent depletion of the transmitter would have to be orders of magnitude more efficient in the relatively large rods of the frog or necturus than in the much smaller rods of man, rat, or cattle. For instance, cGMP phosphodiesterase activities have been reported to be very similar in the rods of frog and cattle,[292] and no other evidence for volume-compensatory mechanisms of any kind have yet been revealed.

The authors of this review are of the opinion that the actual transduction mechanism may involve a number of reactions in the receptor cell which, as yet, we may not even know, and that there is no clear necessity for a diffusable transmitter molecule. The facts that there are more proteins in the disk membrane than previously thought and also that there appear to be a number of enzyme systems in the receptor cell whose presence would not be required by any of the currently discussed hypotheses seem to support this idea.

Such a mechanism could, for instance, make use of one of the special morphological features of the photoreceptor cell, namely, that the pigment-containing membranes form flat, topologically closed compartments with strikingly low permeabilities toward various cations and anions, in particular for H^+. Within these very small compartments (ca. 10^4 times smaller than the cytoplasmic space), very minor changes, induced by the membrane-spanning pigment molecule rhodopsin, could cause major effects. The primary "messenger process" (it does not necessarily have to be a transmitter release), for instance, could take place inside the disk, either in the disk lumen or in the disk membrane itself, and could consequently rapidly reach the rim, from where it would have to be communicated to the plasma membrane. One might then speculate that the rate-limiting step of visual transduction is the communication between rim and plasma membrane and the longitudinal spread of the stimulus in the plasma membrane. This would account for the observed invariability of the time course of single quantum bumps. An interesting observation in this context is the finding of Papermaster et al.[340] that rods of a larger diameter than 1 μm usually have incisions in the disk membrane, accompanied by longitudinal infoldings of the plasma membrane which warrant that no place on the disk surface is more than about 0.5 μm away from the rim and the adjacent plasma membrane.

As to the communication step between disk and plasma membrane, the recently discovered Ca^{2+}/Na^+ exchange system that makes the disk interior accessible from the extracellular space seems to indicate that there is a physical connection between disk and plasma membrane,[22] and one may speculate that it is this connection that bridges the gap between the two membranes.

A great number of experiments, however, will have to be carried out—not only experiments designed to verify existing theories, but also more naive and intuitive experiments aimed at simply collecting data on the complexity of the whole system—before we shall be able to state with confidence which of the current speculations deserve further attention and which level of simplicity we can reasonably expect from nature.

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