Dynamic Processes in Vertebrate Rod Visual Pigments and Their Membranes

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Vision is a transduction process in which light absorbed by a pigment molecule in a photoreceptor cell ultimately produces a transient electrical receptor potential. In terms of the electrical charge movement involved, the process appears to have an amplification factor between 10^4 and 10^5, which rules out any single photochemical or photophysical event as directly responsible for transduction. Instead we must look for a concatenation of events beginning at the molecular level and culminating in changes at the subcellular and cellular level, the whole sequence occurring in a time period of a few milliseconds.

One point which has quite clearly emerged in the more recent chemical and electrophysiological studies of photoreceptor cells is the role of the visual pigment membrane in effecting the transduction process. This membrane is somewhat unique among cellular and subcellular membranes in that the visual pigment, by virtue of its characteristic visible absorption spectrum, provides a built-in probe by which its dynamic properties can be studied. Such studies of visual pigments in their membrane milieu are providing insights not only into the process of vision but also into other sensory processes and are contributing in a major way to our understanding of the functioning of biological membranes in general.

In this Account we examine what is presently known of the dynamic events in visual pigments and their membranes in vertebrate rod cells and how these processes may relate to the transduction process.

The vertebrate rod cell is described in some detail in this issue. For the present Account it suffices to point out that the visual pigment is located in the outer distal segment of the cell. This outer segment has two distinct membrane systems. The visual pigment molecules lie in the surfaces of a number (500–2000) of flattened disc membranes and are oriented so that their spectroscopic transition dipole moments are parallel to these surfaces. The disc membranes form a stack which is ensheathed by a second membrane system, the plasma membrane.

Tomita and his associates[1,2] have shown that illumination of vertebrate photoreceptor cells produces a marked decrease in the dark current flowing in the cell which they assign to a hyperpolarization of the cell. Yoshikami and Hagins[3] and Hagins[4] have studied this phenomenon in some detail and have shown that the apparent normal dark Na^+ current flowing between the inner and outer segments of the cell is blocked by the presence of Ca^{2+} in the external medium in which the cells are immersed. On the basis of the similarity of the hyperpolarization effects of light and Ca^{2+} they hypothesized that the primary action of light was to release Ca^{2+} from the disc membranes; this in turn blocks Na^+ channels in the plasma membrane.

The Visual Pigment Molecule, Rhodopsin

All visual pigments are chromolipoproteins whose chromophores are thought to be derived from only two polyene aldehyde sources, 11-*cis*-retinal and its 3,4-didehydro derivative. Recent X-ray diffraction studies[5] have shown that in the crystal 11-*cis*-retinal has the 12-s-cis form shown in Figure 1a. Rod pigments derived from 11-*cis*-retinal are generally called rhodopsins. The composition of the lipoprotein moiety opsin has not yet been firmly established.[6] It consists of a protein with a highly hydrophobic amino acid content (~50%), but there is some question as to its molecular weight. Both chemical and physical methods[6-15] have yielded values for bovine rhodopsin varying from about 28,000 to 40,000. The predominate value by physical methods is 35,000.[6,11,14]

There is substantial evidence[16-19] indicating that phospholipid is necessary not only to form rhodopsin

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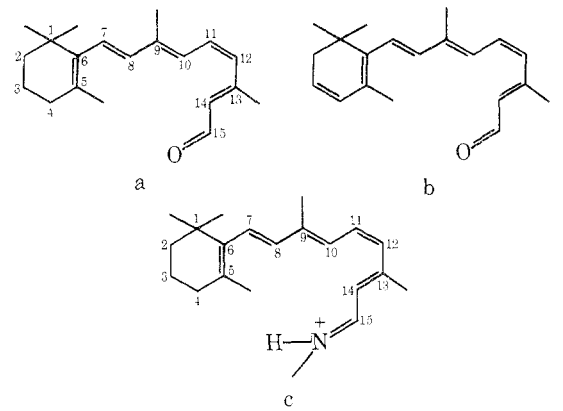

Figure 1. Structural formulas for (a) 11-*cis*-retinal; (b) 11-*cis*-3,4-didehydroretinal; (c) protonated 11-*cis*-retinylidene group, the chromophore of rhodopsin.

from 11-*cis*-retinal and opsin but also to maintain its native conformation. However, the phospholipid components necessary to maintain the conformation do not seem to be sharply defined, although phosphatidylethanolamine[17] appears to be necessary for the formation of rhodopsin and phosphatidylserine appears to be most tightly bound to the protein and most resistant to phospholipase treatment.[20]

The binding of the chromophore to the lipoprotein moiety in vertebrate rhodopsin by chemical[21,22] and spectroscopic[19,23,24] evidence is through a protonated Schiff base. Bownds and Wald[25] demonstrated that the chromophore could be reductively affixed to its binding site when digitonen preparations of bovine rhodopsin were illuminated in the presence of $NaBH_4$. Using this method Bownds[26] and Akhtar[27] identified the chromophore attached to a lysine residue of the protein, presumably at the ϵ-amino group.

Poincelot, *et al.*,[28-30] reduced unilluminated preparations of bovine rhodopsin near 60° with $NaBH_4$ and found the chromophore attached exclusively to the phospholipid, phosphatidylethanolamine. They were also able to extract the chromophore quantitatively as retinylidenephosphatidylethanolamine from unilluminated lyophilized preparations of rhodopsin, but not from illuminated ones. From this evidence they concluded that the chromophore was bound to the phosphatidylethanolamine in native rhodopsin and underwent transimination to the protein upon illumination. Under different sets of conditions, however, other investigators[31-33] found the chromophore distributed between phosphatidylethanolamine and the protein. Further, Hall and Bachrach[33] and Borggreven, *et al.*,[34] described preparations of rhodopsin which apparently had too little phosphatidylethanolamine to completely accommodate the chromophore.

More recently, Fager, *et al.*,[6,35] were able to reduce unilluminated preparations of rhodopsin with $NaBH_3CN$ under conditions which would not denature rhodopsin. They identified the chromophore exclusively attached to the protein. Further, recent resonance Raman spectral studies of bovine rhodopsin preparations by Lewis, *et al.*,[24] show a band at 1645 cm^{-1} which, in position and character, mimics that found for the lysine complexes of retinal rather than that for the phosphatidylethanolamine complex found at somewhat higher energies near 1650 cm^{-1}.

The shape of vertebrate rhodopsin is still not established. Based on X-ray diffraction data Blaisie[36] concluded that frog rhodopsin in the disc membrane is approximately spherical with a diameter of about 42 Å. Electron micrographs indicate that digitonin micelles of rhodopsin are approximately spherical.[6] However, some anisotropy is apparent from the fact that the chromophore can be aligned by shear.[38] A different picture arises from the work of Wu and Stryer[37] on fluorescent labeled rhodopsin in digitonin micelles. From the efficiencies of energy transfer between the labels they concluded that rhodopsin must have a markedly elongated shape with a length of at least 75 Å. With proper alignment they suggested that it could traverse the membrane. It may be that the shape of rhodopsin depends to some extent on labeling and its membrane or micelle environment, but hardly to the extent indicated above. The problem presumably will be solved when and if rhodopsin is crystallized and its structure determined by X-ray diffraction.

The spectra of visual pigments is one of Nature's enigmas. Although protonated Schiff bases of retinal and simple amines in hydroxylic solvents have their long-wavelength visible absorption maxima near 440 nm,[39] visual pigments based on 11-*cis*-retinal are known which vary in absorption maxima from 345 to 375 nm,[6] a range of almost 12,000 cm^{-1}.

Irving, *et al.*,[40] and Blatz[41] found that the visible spectral maxima of protonated retinylidene samples of butylamine depended markedly on the solvent as well as the counteranion. Irving, *et al.*,[40] attributed this to solvent polarizability based on a suggestion of Platt.[42] They reasoned that in the polyene environment of visual pigments groups on the protein with high polarizability such as tryptophan could interact

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with the large transition dipole of the protonated polyene system, thereby producing a substantial bathochromic shift in the spectral maximum. Blatz,[41] on the other hand, attributes these spectral shifts to the delocalization of the positive charge into the polyene chain where it presumably undergoes charge-pair interaction with the counteranion.

Another model, that of a coulombic perturbation of the spectrum by a charged anionic group in the micro environment of the polyene system, was originally suggested by Kropf and Hubbard.[43] this has been examined theoretically in a Pariser–Parr–Pople SCF–MO calculation[6,44] and also by a more elaborate CNDO calculation.[45] Both calculations bear out the notion that a simple coulombic point charge or charged group appropriately placed at different positions relative to the polyene chain can produce bathochromic or hypsochromic shifts sufficient to account for the full range of spectral maxima observed. Further support is given to this model by the fact that Pariser–Parr–Pople calculations comparing the behavior of the N-protonated 11-*cis*-retinylidene group with its 3,4-didehydro derivative are in accord with experimental observations.[44]

Although the anionic perturbation model is consistent with experiment, there are indications from recent resonance Raman spectral studies[24] that aromatic amino acid residues interact with the chromophore and thereby contribute to its spectral perturbation.

The Pigment Disc Membrane

The role of rhodopsin in the neural transduction process should depend on its disposition in the disc membrane. In thoroughly washed fragments of rod outer segments (ROS) rhodopsin constitutes greater than 80% of the membrane protein, although in unwashed ROS it is apparently much smaller.[12,13,19,46] Several unpigmented protein fractions, present in small amounts, have also been detected in ROS.[47]

The lipid of the disc membrane is predominately phospholipid, with only small amounts of cholesterol. This suggests a rather fluid consistency which is also borne out by the analysis of the fatty acid side chains of the phospholipids. These show a very high content of unsaturated fatty acids, the predominant one being docosahexanoic acid, having six unconjugated double bonds.[6,19]

The fluid character of the membrane lipid matrix has been more directly demonstrated by studies on the thermal phase transitions in the vertebrate disc membranes.[48] A sharp reversible thermal phase transition occurs in the lipid matrix of frog discs at about −28°; this contrasts with broad transitions near 0° found in the less fluid mitochondrial and microsomal lipid extracts of rat liver.[49]

The structure of the disc membrane is a problem currently under study in several laboratories. Worthington[50] on the basis of X-ray diffraction studies views the disc membrane as having an essentially lipid bilayer structure but with the greater amount of the high electron density material (protein) on the inner, intradiscal surface of the membrane. Freeze-etch electron microscopy studies by Mason *et al.*[51] appear to support this interpretation. On the other hand similar studies in Hubbell's laboratory[52,53] (see also this issue) indicate that the pigment molecules are in a more hydrophobic environment, and he tends to view rhodopsin as a "transmembrane protein" in support of Stryer's long, rod-like model of rhodopsin. Blaisie,[36] on the basis of his rhodopsin antibody studies, places rhodopsin on the external, interdiscal surface of the membrane. Raubach, *et al.*[54] also conclude that the rhodopsin has some extension into the extradiscal space.

It was implicit in the work of Schmidt[56] and Denton[57,58] that rhodopsin is oriented in the disc membrane such that the principal transition dipole moment of the chromophore is aligned parallel to the disc surface. Hagins and Jennings,[59] however, observed no induced photodichroism when retina were illuminated with polarized light. They suggested that, among other possibilities, this might arise from a rotational diffusion of the chromophore. Brown[60] confirmed their results and further found that photodichroism could be induced in retina treated with glutaraldehyde.

Cone,[61] using flash illumination, observed a transient photodichroism with a relaxation time of 20 μsec. He implied that this arose from a rotational diffusion of rhodopsin about an axis *perpendicular* to the surface of the disc membrane, as would be expected. But he also introduced the possibility of rhodopsin being a transmembrane protein which could act as a carrier by rotating about an axis *parallel* to the membrane surface. However, if this were a normal motion of the rhodopsin in the dark, the rotation axis would have to essentially coincide with the transition dipole moment of the chromophore in order that the latter could remain parallel to the surface, a condition which seems unlikely.

In later papers[62,63] Cone and Poo demonstrated an apparent lateral diffusion of rhodopsin in the membrane. They also[63] described a transmembrane model for rhodopsin which had a dumbbell shape; the two hydrophilic ends protruding from the intra- and

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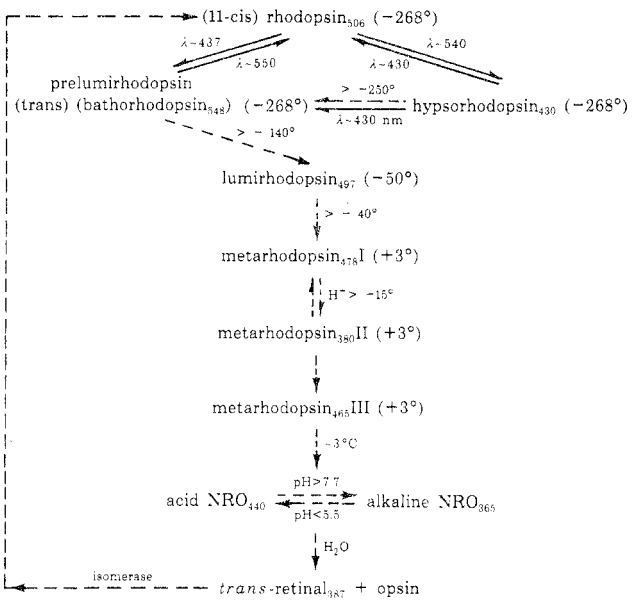

Figure 2. The sequence of intermediates in the photolysis of bovine rhodopsin. Numerical subscripts denote spectral maxima of the long wavelength absorption band. NRO is *N*-retinyldeneopsin.

interdiscal surfaces joined by a hydrophobic portion immersed in the lipid median region. It is difficult, however, to imagine such a structure undergoing rapid rotational diffusion about an axis parallel to the disc surface, as Cone[61] suggested earlier.

Another puzzling feature of the Cone model is that it implies that the polyene chromophore is immersed in the median hydrophobic region of the membrane and still is aligned parallel to the plane of the membrane surfaces, whereas the measurements of Wright and Wald[38] show that the chromophore is essentially parallel to the direction of shear in gel films. If it is assumed that rhodopsin is ellipsoidal or rodlike, the shearing force would be expected to align the molecule with its long axis in the direction of the shear. In the membrane this would position the chromophore perpendicular to its surfaces.

Considering the apparent conflicting evidence, chemical and physical, regarding the shape and disposition of rhodopsin in the disc membrane, it is obvious that much more definitive studies are necessary to clarify these points.

Photodynamic Changes in Rhodopsin and the Rod Photoreceptor

The Photochemistry of Rhodopsin. The photolyses of vertebrate rhodopsin as aqueous suspensions of detergent micelles, whole or fragmented rod disc membranes, excised retina, or intact eyes show essentially the same overall photochemistry.

The sequence of intermediates which have been spectrally identified are shown in Figure 2. There is an initial primary photochemical process involving photoisomerization of the chromophore followed by five dark, thermal reactions terminating in the hydrolytic scission of the chromophore as *trans*-retinal from its lipoprotein moiety, opsin. Quantum yield measurements of 11-*cis*-retinal based pigments in aqueous digitonin suspensions are close to 0.67 in the spectral range 400–650 nm[64] spanning the long-wavelength absorption band (λ_{max} 500 nm). Recent *in situ* measurements of the quantum yield at 504 nm in the frog retina by Baumann[65] also are very close to 0.67.

In the ultraviolet (254–280 nm) the quantum yield as measured by Kropf[66] is 0.21 ± 0.07 at 254 nm, and at 280 nm is 0.26 ± 0.06. He attributes this photosensitivity predominantly to energy transfer from the protein moiety to the chromophore.

Studies of the intermediate processes have been done using low-temperature spectroscopy and flash photolysis techniques (for a review see ref 6). Most pertinent to the physiological process, of course, are those studies carried out near room temperature. The rapidity of the earlier processes have required relaxation techniques capable of monitoring events occurring in the range of nanoseconds. In Figure 2 the sequence of intermediate processes through metarhodopsin380 II is completed in less than a millisecond at physiological temperatures. These processes therefore are logical candidates for involvement in neural transduction. Beyond metarhodopsin380 II the intermediate processes are much too slow for this and are possibly involved in the regeneration of rhodopsin.

The Primary Photochemical Process

Absorption of light in the long-wavelength absorption band of rhodopsin populates the lowest energy singlet π,π^* state to which electronic excitation from the ground state is dipole allowed.[6,19] It is most likely that the primary photochemical process of photoisomerization occurs directly from this state.

Yoshizawa and Kito,[67] by irradiating rhodopsin at liquid nitrogen temperature, obtained the presumed product of the primary photochemical process known as prelumirhodopsin (now renamed bathorhodopsin548).[68]

Yoshizawa[68] has recently identified another possible intermediate, hypsorhodopsin450, by irradiation of rhodopsin at liquid helium temperature at wavelengths longer than 540 nm. This product is converted to bathorhodopsin548 thermally at temperatures greater than −250° or photochemically by irradiation at wavelengths near 406 nm. Irradiation of rhodopsin directly at wavelengths near 437 nm at liquid helium temperature yields bathorhodopsin548. It is quite possible that hypsorhodopsin450 is a true precursor of bathorhodopsin548, but Yoshizawa[68] does not feel that the evidence gathered so far warrants this conclusion.

It seems reasonably certain that the primary photochemical process involves isomerization about the C(11)–C(12) double bond of the chromophore. This is supported by the picosecond laser flash studies of Busch, *et al.*,[55] which show that absorption in the bathorhodopsin band appears in less than 20 psec following photon absorption, which is about what one would expect for the time of isomerization.

The exact conformations of bathorhodopsin548 and hypsorhodopsin450 are still not known. One could

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logically infer that hypsorhodopsin is basically trans about C(11)–C(12) but twisted relative to some single bond. This could explain the pronounced hypsochromic shift in the absorption spectrum relative to rhodopsin and its photochemical and thermal conversion to bathorhodopsin$_{548}$ above −250°. Bathorhodopsin, on the other hand, could exist in a partially twisted form about the C(11)–C(12) double bond.[69]

Thermal Reactions

Metarhodopsin$_{478}$ I → Metarhodopsin$_{380}$ II. This was the first intermediate process whose kinetics were studied by flash photolysis,[70-73] although the intermediates were not so identified until later.[74] A curious feature noted in these early studies in aqueous suspensions of detergent micelles of rhodopsin[72,73] was that metarhodopsin$_{478}$ I appeared to exist in several different forms which were spectrally identical but different in their first-order decay rate constants. This behavior was in contrast to aqueous suspensions of whole or sonicated discs[75] and intact retina[71] whose kinetics usually showed only a single first-order decaying species. The number and decay rates of the multiforms of metarhodospin$_{478}$ I appeared to vary with the mode of preparation and its age and storage conditions.[75]

The process metarhodopsin$_{478}$ I → metarhodopsin$_{380}$ II has a number of interesting chemical features. First of all, Matthews, *et al.*,[74] and Ostroy, *et al.*,[76] showed that an equilibrium could be detected between the two intermediates at temperatures near 0° which was displaced toward metarhodopsin$_{380}$ II by acid. Falk and Fatt[77] and Emrich[78] demonstrated an uptake of one proton in the process. It can also be inferred from phospholipid extraction studies[79] at different intermediate stages that phospholipid is also released in this intermediate process amounting to roughly two molecules of phosphatidylethanolamine and one of phosphatidylcholine. This of course would mean a change in the charge of the opsin moiety. One may write an equation for the process as

$$\text{metarhodopsin}_{478}\ \text{I} + \text{H}_3\text{O}^+ \rightleftharpoons \text{metarhodopsin}_{380}\ \text{II} + n\text{PL} \quad (1)$$

where nPL represents the number of moles of phospholipid released in the process.

Lamola, *et al.*,[80] have shown that the effect of pressure and detergents on the equilibrium shown in eq 1 depends on rhodopsin–phospholipid interactions. The phospholipid content and its interaction with rhodopsin also control the rate of decay of metarhodopsin$_{478}$ I. This is implicit in the studies of Rapp[81,82] who found that sonicated bovine disc membrane suspensions decayed by a single first-order process with a half-life of 0.4 msec at 39° while digitonin preparations exhibited several first-order decay processes, the fastest having a half-life of 27 μsec at comparable temperatures. Presumably these different decay rates arose from differences in the phospholipid and detergent content of the micellated rhodopsin. This point has been recently more explicitly demonstrated by Williams, *et al.*,[83] and Appelbury, *et al.*[80]

Another point of significance in the process metarhodopsin$_{478}$ I → metarhodopsin$_{380}$ II is the magnitude of the activiation parameters.[73,80,81] For bovine rod disc fragments $\Delta H^* = 30.7$ kcal/mol and $\Delta S^* = 55$ cal/mol deg. This suggests that substantial changes are involved in the lipoprotein configuration, but it may be that the phospholipid release and the accompanying charge changes account for these large activation parameters. Clearly, as this process is the only sufficiently rapid process that involves the aqueous environment, *i.e.*, proton uptake, it appears to be the key intermediate process in transduction.

Lumirhodopsin$_{492}$ → Metarhodopsin$_{478}$ I. The kinetics of this process, like the decay of metarhodopsin$_{479}$ I, showed the apparent existence of more than one first-order decaying form.[84] Rapp, *et al.*,[81,82] have shown that the process also fits second-order kinetics but it does not appear to involve chemical reaction with the environment although, as in the decay of metarhodopsin$_{578}$ I some release of phospholipid appears to be involved.[79]

A significant feature of the kinetics of this process is the apparent sensitivity to the viscosity of the medium. Thus, in rod discs, by a first-order treatment over the temperature range −40 to −50°, $\Delta H^* = 25$ kcal/mol and $\Delta S^* = 70$ cal/mol deg, while above 3° $\Delta H^* = 3.5$ kcal/mol and $\Delta S^* = -5.8$ cal/mol deg. In glycerol suspensions of digitonin micelles, the process at low temperature is about two orders of magnitude slower than in rod discs. Its half-life in rod discs at 26° is approximately 18 μsec. As previously pointed out there is a thermal phase change in the lipid matrix of the visual pigment membrane near −30°.[48] This could account for the large differences in the ΔH^* and ΔS^* at low and high temperatures and probably indicates a different rate-controlling step in the process in the two temperature ranges.

Bathorhodopsin$_{548}$ → Lumirhodopsin$_{492}$. This is the fastest of the three early intermediate reactions. Like the others, it has also exhibited the multiform first-order decay process in digitonin micelles[85] and, apparently, even in rod particles[86] at low tempera-

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tures. At room temperature in aqueous lauryldiamine oxide (LDAO) suspensions only a single decay rate is apparent with an extrapolated half life of about 10 nsec at 38°.[55] The calculated activation parameters near this temperature are $\Delta H^* = 6.0$ kcal/mol and $\Delta S^* = 15$ cal/mol deg, which are in essential agreement with those obtained near −100°.[85,86] These are consistent with the view that the process is a simple relaxation, perhaps involving a thermal isomerization about a formal single bond of the chromophore. However, the multiform character of the reaction, assuming it is not artifact, suggests that it may be sensitive to bound phospholipid.

The Decay of Metarhodopsin$_{380}$ II

It has already been pointed out that the processes following metarhodopsin$_{380}$ II are too slow for involvement in neural transduction. Their exact role in vision is unknown, but it is likely that they are involved in the regeneration of rhodopsin.

The intermediate sequence shown in Figure 2 shows metarhodopsin$_{380}$ II decaying to metarhodopsin$_{465}$ III, called pararhodopsin by Wald.[87] Ostroy, *et al.*,[88] conclude that at least one sulfhydryl group is exposed in this process. Metarhodopsin$_{465}$ III decays to the acid form of *N*-retinylideneopsin (NRO_{440}), an intermediate in which the Schiff base linkage is directly exposed to the aqueous environment. Like the decay of metarhodopsin$_{478}$ I, it is acid catalyzed and has large activation parameters.[76]

It should be pointed out that another path for the decay of metarhodopsin$_{380}$ II has been proposed by Matthews, *et al.*,[74] in which metarhodopsin$_{380}$ II decays directly to retinal and opsin; apparently both pathways are found in vertebrate retina.[90] The kinetics of these processes have been reviewed elsewhere.[69,89]

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Active Pumping and Release of Calcium Ion by the Disc Membranes

An attempt has been made by Mason *et al.*[90] to test the Yoshikami–Hagins hypothesis by $^{45}Ca^{2+}$ tracer studies. It was found that frog disc membranes or vesicles prepared from these membranes by sonication and resealing could accumulate $^{45}Ca^{2+}$ in the dark against a Ca^{2+} gradient. Furthermore, the rate of Ca^{2+} uptake was almost three times greater for light-adapted membranes than for dark-adapted membranes. Although soluble endogenous material was necessary for the active pumping of Ca^{2+}, it could be largely replaced by MgATP. As expected, the active pumping was inhibited by ouabain.

Using a preparation of sonicated membrane vesicles containing $^{45}Ca^{2+}$ illumination was found to rapidly release Ca^{2+} from the vesicles. Furthermore, a comparison of the number of rhodopsin molecules photolyzed with Ca^{2+} released indicated a 1:1 ratio of the two when 1% or more of the rhodopsin was photolyzed. On the other hand, Szuts (see ref 63) has found that the rod disc membranes themselves release up to one-half their content of Ca^{2+} when only 2 to 4 molecules of rhodopsin are photolyzed. He estimated that the photolysis of a single rhodopsin molecule in a disc membrane releases 10^4 to 10^5 calcium ions. More recently he and Cone[91] have revised this figure to between 10 and 1000 Ca^{2+} per rhodopsin molecule photolyzed corresponding to photolysis levels of 1% and 0.01%, respectively.

In the experiments of Mason *et al.*[90] the photolytic release of Ca^{2+} appeared to be stepwise, *i.e.*, upon partial photolysis of the vesicles a certain amount of intravesicular Ca^{2+} was released and no subsequent Ca^{2+} efflux occurred until further photolysis. This was apparently not observed by Szuts and Cone. At the present time there seems to be no way of reconciling the two sets of results other than to assume that whole discs and their sonicated vesicles use different mechanisms for Ca^{2+} release.

Whatever the number of calcium ions released per rhodopsin photolyzed or the mechanism, the release is certainly rapid and could be associated with the metarhodopsin$_{478}$ I → metarhodopsin$_{380}$ II process.[90]

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