Bioorganic studies with rhodopsin

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<u>Abstract</u> - An introductory review of visual transduction is
followed by an account of external point charge models which were
proposed to explain the red-shifted maxima in visual pigments and
bacteriorhodopsin. 13-Ethylretinal, 13-propylretinal, and a
retinal analog with an 8-membered ring in the side-chain yield
bovine rhodopsin analogs which are bleachable but spontaneously
regrow. This is a phenomenon not seen before <u>in vitro</u> in
rhodopsins. The photoreceptor of the phototactic green algae
<u>Chlamydomonas</u> has been found to be closely related to bovine
rhodopsin but unlike bacteriorhodopsin; incubation of
<u>Chlamydomonas</u> with retinal analogs shifts the phototaxis action
spectral maxima in a manner similar to the shifts encountered in
the bovine visual pigment. This is the first behavioral shift
induced by analogs in a eukaryote. The studies also suggest that
<u>Chlamydomonas</u> provides a convenient system for photoreceptor
studies because of the availability of mutant strains, relative
ease of experiment, and the short time needed to get results on
minuscule amounts of analogs.

INTRODUCTION

In 1933 Wald discovered that vitamin A was present in the retina (Ref. 1),
and then in 1950 determined that the chromophore of the visual pigment
rhodopsin is 11-<u>cis</u>-retinal (Ref. 2). Studies in vision have dramatically
increased in recent years since these discoveries, and involve researchers
in all branches of science. However, it appears that the more we study,
the more complex the phenomenon of visual transduction becomes. Intimate
collaboration between scientists in all disciplines is indispensable.

An introductory account of visual pigments is given (Refs. 3-6) and this is
followed by unpublished studies. The visual pigments are in the
photoreceptor cells. There are two types of photoreceptors, the rod/cone
cells of vertebrates and the rhabdomeric cells of invertebrates. Both
consist of inner and outer segments, and it is the latter which contain the
photoreceptors. In the vertebrate eye, the light enters the lens (Fig. 1)
and hits the neural photoreceptor cells distributed in the retina. There
are two types of photoreceptors, the rod cells which are primarily in the
peripheral region of the retina, and the cone cells which are clustered
around the central fovea where focusing of image is sharpest. The rod
outer segment (ROS) contains 1,500 stacked disks; the rhodopsin molecules,
λ_{max} 500 nm, which are responsible for black and white sensation in dim
light are embedded in the disks (Fig. 1). The human retina has three types
of cone cells which absorb at 450, 530 and 560 nm and work under bright
illumination. Most animals have only one kind of visual pigment and cannot
distinguish color. Rhodopsin, a hydrophobic protein comprised of 348 amino
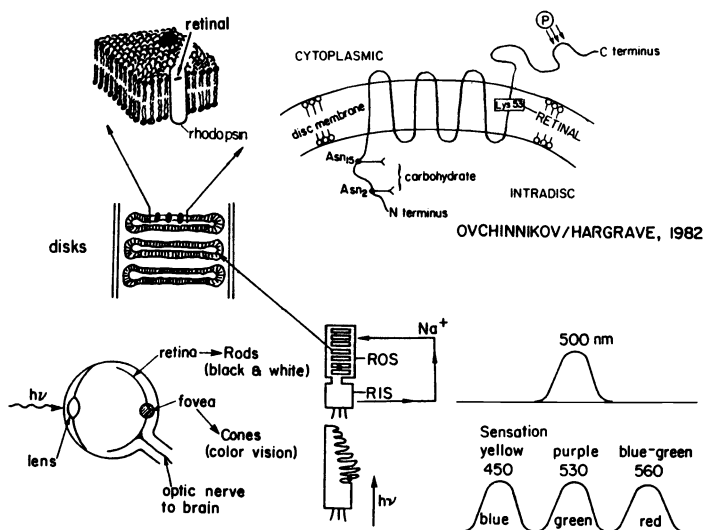acids, traverses the disk membrane several times. Its primary structure

Fig. 1. The eye and photoreceptor cells. The rhodopsin
molecule traverses the disk membrane (upper left and right)

was elucidated recently (Refs. 7, 8) but the tertiary structure (Ref. 9)
has yet to be clarified experimentally.

Vitamin A, produced by cleavage of carotenoids, is partly converted into
11-cis-retinal; binding of this chromophore to the rod and cone opsins
produces the various visual pigments (Fig. 2). Certain animals such as
fish and tadpoles have the 3-dehydro chromophore in addition. Most
rhodopsin studies including those described below are carried out with the
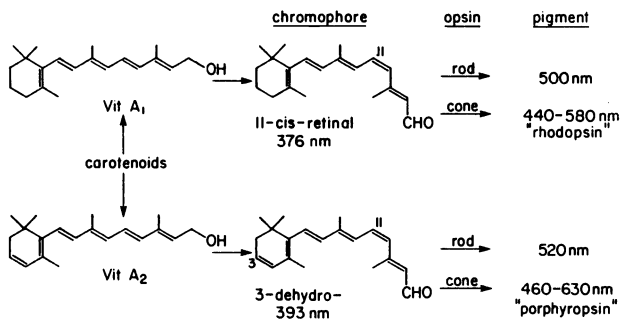bovine pigment because of its availability.

Fig. 2. Formation of visual pigments

The chromophore is bound to lysine 53 via a protonated Schiff base (Fig. 3,
1). Although the bleaching process appears to differ somewhat from animal
to animal, it is generally agreed that the primary photoproduct is
bathorhodopsin discovered in 1958 (Ref. 10). Subsequent steps are thermal
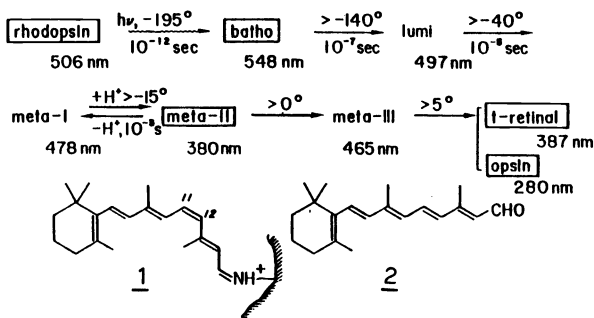and proceed through several intermediates terminating in trans-retinal 2

Fig. 3. Bleaching process of cattle
rhodopsin ($\underline{1}$ = protonated Schiff
base, SBH^+)

and opsin. The light isomerizes the 11-cis-retinal moiety 1 to an unidentified retinal species contained in bathorhodopsin (Ref. 11) within a few picoseconds (Refs. 12, 13). Bathorhodopsin, a ground state species with 35 kcal higher energy than rhodopsin (Ref. 14), possibly stores its energy by separation of the positively charged iminium nitrogen from its counter-anion (hence the red-shift in absorption) (Ref. 15) and by internal strains within the chromophore.

The species directly involved in visual transduction is thought to be metarhodopsin-II. In the dark, sodium ions flow into the rod outer segments (ROS, Fig. 1) from the extracellular fluid and diffuse into the inner segment (RIS) where they are pumped out again by an ATP-dependent pump. Upon illumination, the sodium channel is blocked and the dark current is shut off. This leads to a neural signal which is transmitted to the brain via the optic nerve, but the manner in which the change in shape of rhodopsin, which is triggered by the 11-cis to trans isomerization, relates to transduction is still unclear. Two hypotheses have been forwarded (Ref. 16). Since calcium flows out of the disk into the plasma membrane upon irradiation and since raising the calcium level shuts down the dark current, it has been proposed that calcium is the internal transmitter which stops the flow of sodium ions. In another theory, cyclic GMP is the transmitter. Namely, activated rhodopsin (meta-II) changes a protein called G-protein (or transducin) from an inactive to an active form, and this activates a phosphodiesterase which hydrolyzes cyclic GMP to GMP; the bleaching of one molecule of pigment results in the destruction of 10,000 molecules of cyclic GMP. An understanding of the visual transduction process on a molecular structural basis is one of the central problems in vision science.

EXTERNAL POINT CHARGE MODELS

The factors involved in regulating the absorption maxima of rhodopsins has been a question of major interest. Namely, the protonated Schiff base (SBH$^+$) of retinal with n-BuNH$_2$ absorbs at 440 nm (22,700 cm^{-1}, in MeOH) while the SBH$^+$ in visual pigments absorbs at 440-650 nm (22,700-15,400 cm^{-1}). In bacteriorhodopsin, a light-induced proton-translocating pigment attracting great interest since its discovery in the early 70's (Ref. 17), the SBH$^+$ now absorbs at 560 nm (17,860 cm^{-1}). What are the factors leading to these red shifts? Based on experiments with dihydroretinals we proposed the so-called external point-charge models for the two pigments (Fig. 4, Ref. 18). First the red shifts (in cm^{-1}) in going from 11-cis-retinal, SBH$^+$ with n-BuNH$_2$, 440 nm or 22,700 cm^{-1}, to the pigments, e.g., bovine rhodopsin, 500 nm or 20,000 cm^{-1}, were defined as "opsin shifts" (OS); in
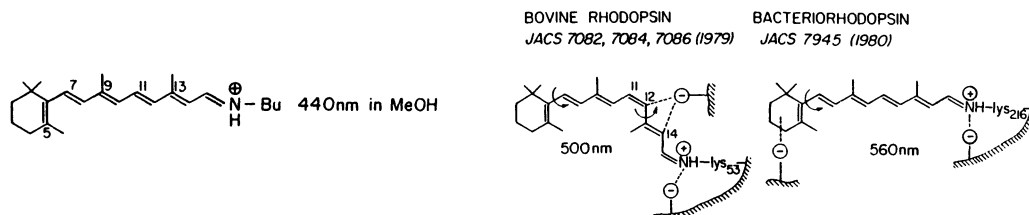

Fig. 4. External point charge models

the example cited it is 2,700 cm^{-1}. The OS is a value which reflects the difference between the environment of SBH$^+$ in methanol and within the binding site.

Our approach in the studies of visual pigments and the closely related bacteriorhodopsin has been to synthesize retinal analogs with modified structures, bind them to apoproteins to produce pigment analogs, and then to clarify certain aspects of natural pigments from the properties of analogs. One series of studies is the following. In the dihydroretinal series (Fig. 5) it is seen that OS has the largest value of 5,300 cm^{-1} for
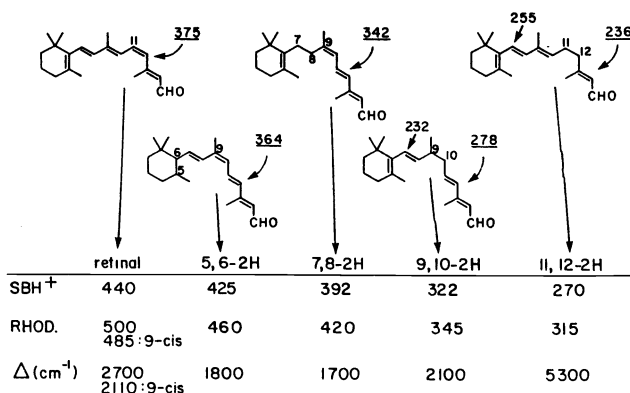
Fig. 5. Opsin shifts of bovine rhodopsins and dihydrorhodopsins

	retinal	5,6-2H	7,8-2H	9,10-2H	11,12-2H
SBH$^+$	440	425	392	322	270
RHOD.	500 485:9-cis	460	420	345	315
Δ(cm^{-1})	2700 2110:9-cis	1800	1700	2100	5300

11,12-dihydroretinal, the analog with the shortest chromophore (Ref. 19). Theoretical treatment of this trend (Ref. 18) coupled with further model studies (Ref. 20) led to the models shown in Fig. 4. Although the validity of these models and the identity of the external charges will not be known until the tertiary structures are clarified, they are currently the generally accepted models.

SPONTANEOUS REGROWTH OF BLEACHED VISUAL PIGMENTS

The study of visual pigments containing retinal chromophores with multiple alkyl substituents in the side-chain is likely to clarify the steric environment of the binding site; also, the extra substituents may decelerate or accelerate the formation of bathorhodopsin and other intermediates. Likewise, bacteriorhodopsin; a comparison of the proton translocation efficiency of the alkylated analogs with natural bacteriorhodopsin could possibly contribute to our understanding of this very important phenomenon. With this in mind a series of alkylated retinals has been synthesized (Ref. 22) and are being bound to form pigments. The hindered retinals were prepared according to Fig. 6 (Ref. 21); the advantage of this method is that it yields the otherwise
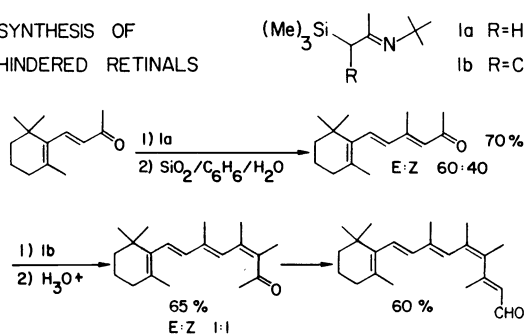
Fig. 6. Synthesis of hindered alkyl retinals
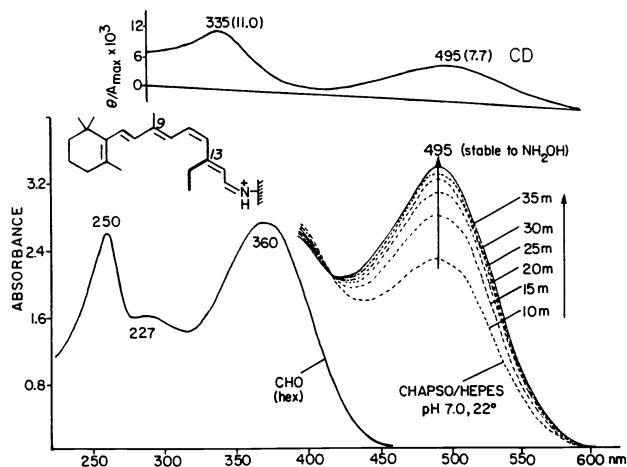
Fig. 7. UV/MS and CD spectra of 13-ethyl-11-<u>cis</u>-retinal and rhodopsin analog

difficult to obtain, severely hindered Z isomers in fair yield. During the course of these studies a unique spontaneous regeneration was encountered. 13-Ethylretinal with maxima at 250, 277, and 360 nm (Fig. 7) readily forms a pigment in the detergent CHAPSO, pH 7.0. It absorbs at 495 nm, and exhibits two positive Cotton effects in the CD spectrum. Upon irradiation with light longer than 520 nm, it "bleaches" in one minute (Fig. 8); however, a spontaneous pigment regrowth is observed in the dark. After 48 hours it has a 485 nm maximum. The pigment from 13-propylretinal also behaves similarly.
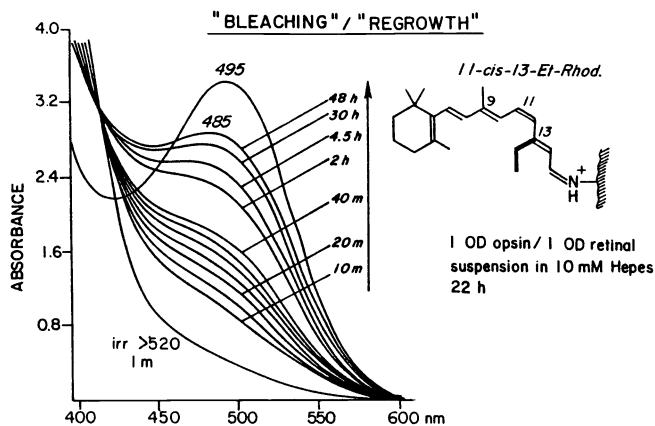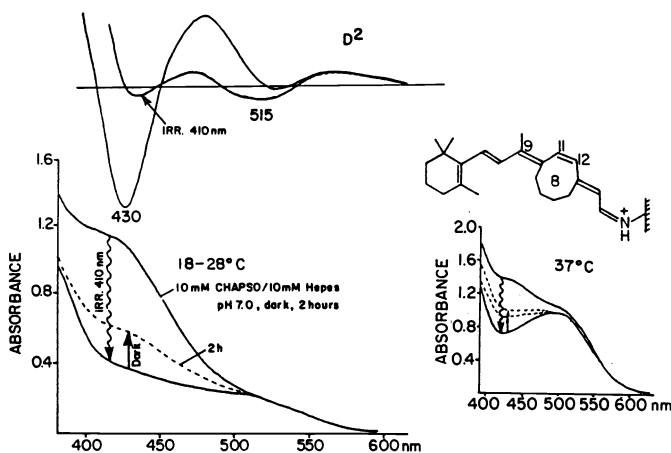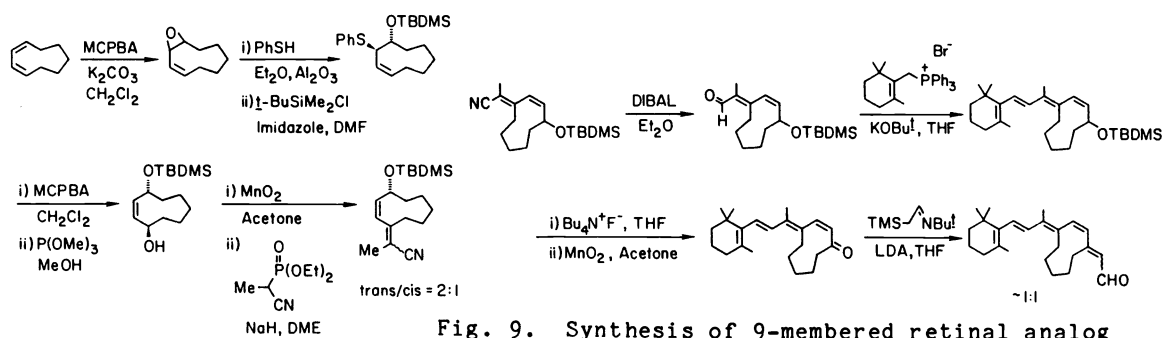
Fig. 8. "Bleaching" and regeneration of a pigment analog from 13-ethylretinal

This regrowth phenomenon can tentatively be interpreted as follows. The maximum of the regenerated species is at the long wavelength of 485 nm and therefore is best explained by an SBH$^+$ species; the "bleached" species on the other hand cannot be SBH$^+$ because of the lack of long wavelength absorption. Irradiation causes a double bond isomerization, but unlike natural 11-<u>cis</u>-retinal, the analog is not freed from the binding site because of the 13-Et or 13-Pr groups, i.e., the extra length of the alkyl group fits into a hydrophobic site thus preventing the SB linkage from being exposed and hydrolyzed. Namely, the C=N bond simply becomes deprotonated, and hence the blue-shift, or "bleaching"; the isomerized form represents an unstable ground state so that it reisomerizes to a stabler form and becomes reprotonated to an SBH$^+$ absorbing at 485 nm.

The 7-membered retinal analog with a fixed 11-ene (Fig. 12) as expected produced a nonbleachable pigment <u>in vitro</u> (Refs. 13, 23) and <u>in vivo</u> (rat

and bull-frog, Ref. 24), thus showing that an 11-<u>cis</u> to 11-<u>trans</u> isomeriza-
tion is a prerequisite for visual transduction. If it were possible to
produce pigment analogs with 8- and 9-membered rings bridging across C-10
and C-13, then: (i) the larger ring may accommodate a <u>trans</u>-11-ene and thus
provide a mimic for bathorodopsin which is generally believed to contain a
highly strained transoid double bond (Ref. 25); and (ii) constraints from
the ring may produce a bleachable pigment which may serve as a mimic for an
intermediate further along the bleaching process (Fig. 3).

The 8- and 9-membered retinal analogs were prepared according to Fig. 9
(Ref. 26). The 8-membered analog appears to give rise to two pigments
(Fig. 10, Ref. 27). Thus when incubated with bovine opsin at 18-28° a
species with a maximum at 430 nm is the major product; at 37° a species

Fig. 9. Synthesis of 9-membered retinal analog

Fig. 10. Pigment formation with
8-membered retinal analog;
derivative spectra are also shown

absorbing at 515 nm is clearly produced. Upon irradiation, bleaching of
the 430 species and subsequent spontaneous regrowth occurs, but the 515
species is not bleached. The 9-membered retinal also gives a pigment,
absorption maximum 407 nm in CHAPSO, which is now bleachable but does not
regrow. Low-temperature spectroscopic studies and checking of enzymatic
activations induced by bleaching and/or regrowth processes of the pigments
formed from alkylated and medium-ring-containing retinals are being planned.

CHLAMYDOMONAS, A NEW MODEL FOR THE STUDY OF PHOTORECEPTORS

Chlamydomonas (Fig. 11) is a 10 μm diameter aquatic organism with a single
eye and two flagella which together lead to phototaxis (Ref. 28). It is
widely used for studying circadian rhythm, flagella architecture and
function, herbicide development, gametogenesis, photosynthesis, etc.; in

collaboration with Prof. K.W. Foster, we have shown that this genetically well-studied eukaryote provides a convenient model for photoreception studies (Ref. 29).

Retinal analogs have given numerous rhodopsin and bacteriorhodopsin analogs absorbing at different wavelengths. At least 4 analogs have been shown to shift the behavioral action spectrum of Halobacterium (Ref. 30). In the following a blind mutant in which carotenoid biosynthesis is blocked at a step preceding phytoene was employed. The mutant is negatively phototactic, i.e., it swims away from light. If a retinal moiety were the chromophore, addition of retinal to the cell suspension of vegetative cells should restore phototaxis. Indeed it was found that when left overnight so that cells became gametes and incorporate the retinal, the phototaxis behavior was restored. Moreover, it was found that addition of dihydroretinals induced phototaxis with action spectra maxima closely
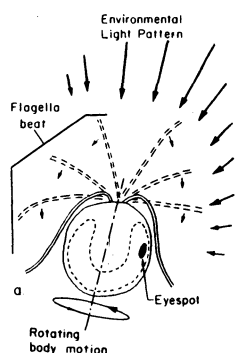

Fig. 11. Chlamydomonas

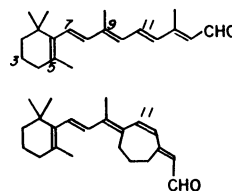|	Rh	Chlamy.	bR
1) t-retinal		501	560
2) 3,4-de-H		511	600
3) 11c	500	505	
4) 9c	485	488	
5) 9c-5,6-2H	460	467	476 ✝
6) 7,8-2H		434	400 ✝
7) 9c-7,8-2H	420	438	
8) 7-memb.	490	X	

Fig. 12. Induced photoaxis in Chlamydomonas and maxima of rhodopsin, bacteriorhodopsin and analogs

resembling the absorption maxima of bovine rhodopsin but unlike those of bacteriorhodopsin (Fig. 12; see also Fig. 5). The natural action spectral maximum of Chlamydomonas is at 503 nm, whereas the maxima after trans and 11-cis-retinal incorporation are at 501 and 505 nm, respectively. Since these values are within experimental error it is likely that the retinals are isomerized in the dark (to 11-cis or 9-cis or trans). However, the 488 nm maximum caused by incorporation of the 9-cis isomer is outside the range of error and parallels the 485 nm of bovine pigment. These findings lead us to suspect that Chlamydomonas, an organism which separated from our evolution line about 1 billion years ago, has a pigment similar to the bovine rhodopsin (Ref. 29).

Irradiation of the blind mutant leads to restoration of natural phototaxis. Furthermore, incorporated retinal analogs also restore native phototaxis when irradiated around their respective maxima, e.g., 7,8-dihydroretinal, which leads to a phototaxis action spectral maximum at around 435 nm (Fig. 12) induces native phototaxis most efficiently when irradiated at 435 nm. This shows that retinals play a dual role, one is to cause phototaxis and the other is to turn on the blocked carotenoid biosynthesis (Ref. 31). These are the first demonstrations of a rhodopsin photoreceptor in an alga or eukaryotic protist and also the first behavioral spectral shifts caused by exogenous synthetic retinals in a eukaryote.

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