10,20-Metanorhodopsins: (7E,9E,13E)-10,20-methanorhodopsin and (7E,9Z,13Z)-10,20-methanorhodopsin: 11-cis-locked rhodopsin analog pigments with unusual thermal and photo-stability

Grip, W.J. de; Oostrum, J. van; Bovee Geurts, P.H.M.; Steen, R. van der; Amsterdam, L.J.P. van; Groesbeek, M.; Lugtenburg, J.

Citation

Grip, W. J. de, Oostrum, J. van, Bovee Geurts, P. H. M., Steen, R. van der, Amsterdam, L. J. P. van, Groesbeek, M., & Lugtenburg, J. (1990). 10,20-Metanorhodopsins: (7E,9E,13E)-10,20-methanorhodopsin and (7E,9Z,13Z)-10,20-methanorhodopsin: 11-cis-locked rhodopsin analog pigments with unusual thermal and photo-stability. *European Journal Of Biochemistry*, *191*(1), 211-220. doi:10.1111/j.1432-1033.1990.tb19112.x

Version: Publisher's Version

License: Licensed under Article 25fa Copyright Act/Law (Amendment Taverne)

Downloaded from: https://hdl.handle.net/1887/3281563

Note: To cite this publication please use the final published version (if applicable).

Eur. J. Biochem. *191*, 211 – 220 (1990)
© FEBS 1990

10,20-Methanorhodopsins: (7*E*,9*E*,13*E*)-10,20-methanorhodopsin and (7*E*,9*Z*,13*Z*)-10,20-methanorhodopsin

11-*cis*-Locked rhodopsin analog pigments with unusual thermal and photo-stability

Willem J. de GRIP[1], Jenny van OOSTRUM[1], Petra H. M. BOVEE-GEURTS[1], Rob van der STEEN[2], Leen J. P. van AMSTERDAM[2], Michel GROESBEEK[2] and Johan LUGTENBURG[2]

[1] Department of Biochemistry, University of Nijmegen, The Netherlands
[2] Department of Organic Chemistry, Gorlaeus Laboratory, University of Leiden, The Netherlands

(Received September 15, 1989/February 28, 1990) – EJB 89 1125

Synthesis of the retinal analog, 10,20-methanoretinal (R6), where the 11*Z* conformation is locked in a six-membered ring, yielded four stereoisomers (7*E*,9*E*,13*E*; 7*E*,9*E*,13*Z*, 7*E*,9*Z*,13*E* and 7*E*,9*Z*,13*Z*). These four isomers were separated by straight-phase isocratic HPLC and identified by ^{1}H-NMR and NOE analysis. All isomers smoothly recombined with bovine opsin at a relatively high rate (5 – 10% of that of the natural chromophore 11*Z*-retinal). The corresponding 13*E* and 13*Z* isomers yielded identical analog pigments, probably due to rapid thermal isomerization around the C13 = C14 double bond. The (7*E*,9*E*)-isomers produced a pigment with maximal absorbance at 510 nm, while the pigment produced from the (7*E*,9*Z*)-isomers had maximal absorbance at 494 nm. Based upon kinetic considerations, the chromophore structure in the 510-nm-absorbing pigment should be (7*E*,9*E*,13*E*), i.e. equivalent to 11*Z*-retinal and rhodopsin, while the chromophore structure in the 494-nm-absorbing pigment should be (7*E*,9*Z*,13*Z*), i.e. equivalent to (9*Z*,11*Z*,13*Z*)-rhodopsin, an isorhodopsin analog. In analogy to the 11-*cis*-locked rhodopsin analogs Rh5 and Rh7, the 510-nm-absorbing pigment, (7*E*,9*E*,13*E*)-10,20-methanorhodopsin, was dubbed Rh6 and the 494-nm-absorbing pigment, (7*E*,9*Z*,13*Z*)-10,20-methanorhodopsin, was dubbed Iso6. The opsin shift of Rh6 (2660 cm^{-1}) is practically identical to that of rhodopsin itself (2650 cm^{-1}). Rh6 and Iso6 are nearly as stable as rhodopsin towards hydroxylamine and solubilization in detergent solution and could be easily purified and reconstituted into proteoliposomes by established procedures. Due to the 11-*cis*-lock, Rh6 is much less photolabile (bleaching rate < 1%) than rhodopsin, but it is not completely photostable, probably since photoisomerization around the C7 = C8, C9 = C10 and C13 = C14 bonds is allowed. Illumination of either Rh6 or Iso6 does not generate the common photointermediates but instead produces a complex pattern of photochemical transitions, which during continuous illumination leads to the same final steady state, absorbing at 498 nm. This process is accompanied by a slow but steady loss of pigment, probably due to hydrolytic release of chromophore, which is markedly accelerated in the presence of hydroxylamine. In a physiological assay (light-dependent activation of rod cGMP phosphodiesterase) Rh6 is only marginally active and this probably reflects conformational changes accompanying the above-mentioned photochemical transitions. This supports the concept that normal rhodopsin-based phototransduction requires 11*Z* to all-*E* isomerization. Complete photostability and physiological inactivity could be achieved by substituting the Schiff-base link in Rh6 by an amide link, which is much less susceptible to hydrolytic cleavage, i.e. by recombining bovine opsin with (7*E*,9*E*,13*E*)-10,20-methanoretinoyl fluoride.

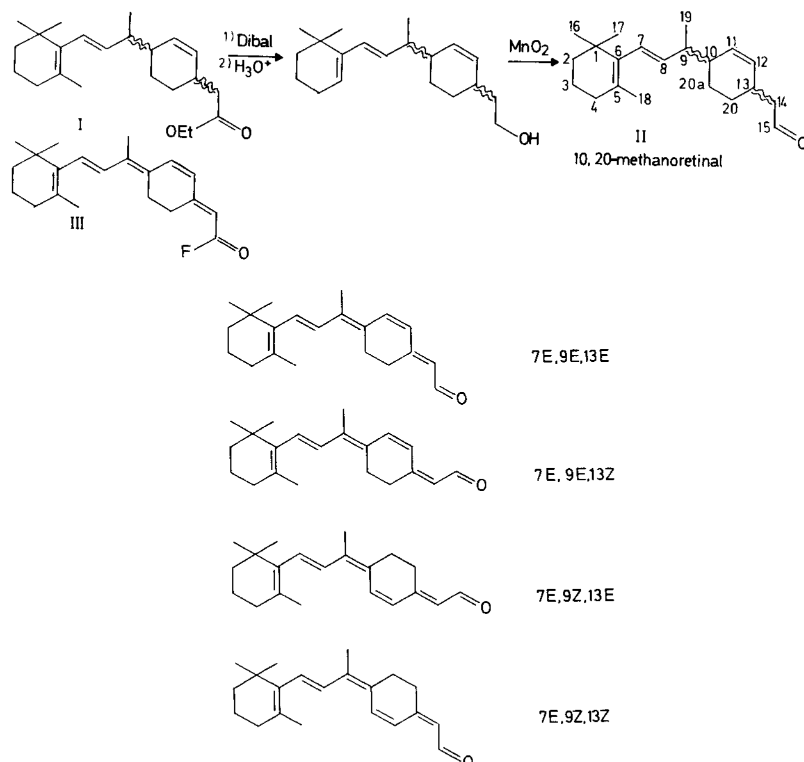
These results demonstrate that the six-membered ring 11-*cis*-locked rhodopsin analog pigments Rh6 and Iso6 are spectrally and structurally highly akin to rhodopsin, but lack its high photosensitivity and physiological activity. They could serve as suitable controls for rhodopsin in studies towards its light-triggered functions or as suitable alternatives in studies on those properties which are not light-dependent.

The use of synthetic analogs of 11-*cis*-retinal to study its binding site in, and its mode of interaction with, the visual pigment rhodopsin, as well as to analyse the geometric and steric requirements of the photolytic cascade triggered upon absorption of light by rhodopsin, has yielded valuable structural and functional information [1 – 4].

In order to investigate the requirement of 11-*cis* to all-*trans* isomerization (alleged to be absolute) for photocascade and hence function of rhodopsin, synthesis of two 11-*cis*-locked retinal analogs has been described, which lock the 11-*cis* conformation in a 7-membered ring (10,20-ethanoretinal, R7 [5]) or a 5-membered ring (10,20-cycloretinal, R5 [6]). Upon incubation with bovine opsin both compounds slowly regenerated the analog pigments (7*E*,9*E*,13*E*)-10,20-ethanorhodopsin (Rh7) and (7*E*,9*E*,13*E*)-10,20-cyclorhodopsin

Correspondence to W. J. de Grip, University of Nijmegen, Department Biochemistry, P.O. Box 9101, NL-6500 HB Nijmegen, The Netherlands

Abbreviations. Rh6, (7*E*,9*E*,13*E*)-10,20-methanorhodopsin; Iso6, (7*E*,9*Z*,13*Z*)-10,20-methanorhodopsin; RhIII, (7*E*,9*E*,13*E*)-10,20-methanoretinoyl opsin; Rh5, (7*E*,9*E*,13*E*)-10,20-cyclorhodopsin; Rh7, (7*E*,9*E*,13*E*)-10,20-ethanorhodopsin; R5, 10,20-cycloretinal; R6, 10,20-methanoretinal; R7, 10,20-ethanoretinal; G-protein, GTP-binding protein or transducin.



Scheme 1. *Structures of the six-membered ring 11-*cis*-locked retinal analogs used in this work.* Formula II represents the general structure of 10,20-methanoretinal (R6) and is prepared from the corresponding ethylester I via reduction with diisobutylaluminum hydride (Dibal) to the alcohol, followed by oxidation with MnO_2 using standard conditions [1, 2, 16]. This yields a mixture of the four *E/Z* stereoisomers of R6 shown below. The synthesis of the ester I and the acyl fluoride III has been described in detail before [16]

(Rh5) with λ_{max} 490 nm [5] and 495 nm [6, 7], respectively. Both analog pigments were indeed shown to be photostable, i.e. they did not produce a bathorhodopsin analog or subsequent photointermediates upon illumination [8–10]. Nevertheless, prolonged illumination of Rh5 established a photoequilibrium between two, probably configurationaly slightly different, analog pigments [7], and incorporation of the 7-membered analog into a blind mutant strain of *Chlamydomonas reinhardtii* effectively restored its phototaxis [11, 12]. On the other hand, supplementation of vitamin-A-depleted rats with the same analog produced analog pigment without recovering photosensitivity [13].

We were interested in investigating the functionality of 11-*cis*-locked rhodopsin analogs in more detail and, in addition, our studies on rhodopsin crystallization could benefit from an analog which is photostable and structurally closely related [14]. However, these approaches require reasonably thermostable analog pigments which survive detergent solubilization, purification etc. Unfortunately, both Rh5 and Rh7, already in the membrane-bound form, were not stable towards hydroxylamine and already slowly decomposed in the very mild detergent digitonin [5–7]. Hence, we searched for alternatives. Longer ring systems seemed also to be inappropriate [2]. The intermediate system, a 6-membered ring, was reported not to generate an analog pigment [15]. However that particular retinal analog also lacked the 19-methyl substituent. Hence, we investigated the potential of the true 6-membered 11-*cis*-locked retinal analog, 10,20-methanoretinal (R6).

Here we describe the synthesis, purification and spectroscopic properties of four geometric isomers of this analog (11*Z*; 11*Z*,13*Z*; 9*Z*,11*Z* and 9*Z*,11*Z*,13*Z*) and their interaction with bovine opsin. The corresponding 13*E* and 13*Z* isomers yield the same analog pigment, probably due to rapid thermal isomerization of the C13 = C14 double bond. The resulting analog pigments (7*E*,9*E*,13*E*)-10,20-methanorhodopsin (λ_{max} 510 nm) obtained from the 11*Z*-isomer and (7*E*,9*Z*,13*Z*)-10,20-methanorhodopsin (λ_{max} 494 nm) obtained from the (9*Z*,11*Z*,13*Z*)-isomer, were dubbed Rh6 and Iso6, respectively. They present the first class of 11-*cis*-locked rhodopsin analogs which are nearly as stable as the parent rhodopsin towards hydroxylamine and common detergents and which can be purified by common purification strategies.

MATERIALS AND METHODS

Materials

10,20-Methanoretinal (R6, compound II in Scheme 1) was prepared from ethyl 10,20-methanoretinoate (I) by reduction with diisobutylaluminum hydride (dibal) to the corresponding alcohol, followed by oxidation to the aldehyde (Scheme 1). This afforded compound II as a mixture of four isomers (7*E*,9*E*,13*E*; 7*E*,9*E*,13*Z*; 7*E*,9*Z*,13*E* and 7*E*,9*Z*,13*Z*). The individual compounds could be purified by straight-phase isocractic HPLC (Fig. 1). The isomeric configuration of the purified fractions was established by ^{1}H-NMR analysis (Table 1). Relevant spectroscopic data are collected in Table 2. The preparation of compound I as well as the corresponding fluoride III has been described in detail before [16].

O-Nonyl β-1-glucoside was prepared and purified as described [17] except that straight-phase chromatography was performed in the preparative HPLC mode using stepwise elu-

tion with 1 – 5% (step = 1%) methanol in dichloromethane. *O*-Dodecyl *β*-1-maltoside was prepared and purified as described [17] or obtained from Sigma (St Louis, USA). All other chemicals were of the highest grade available.

Preparation of analog pigments

Bovine rod outer segment membranes in the opsin form (opsin membranes) were prepared from fresh dark-adapted eyes according to described procedures (mild homogenization of the retina; filtration; sucrose-density gradient centrifugation; low-speed centrifugation; lysis; high-speed centrifugation) performed in the light [18, 19]. The final preparations were stored as a pellet at –80°C. The regeneration capacity of these preparations was estimated from the A_{280}/A_{500} ratio obtained upon subsequent incubation with a threefold excess of 11-*cis*-retinal, whereby a ratio of 2.0 was taken to represent membranes with the maximal rhodopsin content [18]. Most preparations exhibited a regeneration capacity in the range 90 – 100%. Incidental preparations with lower capacity were not used to generate analog pigments. All manipulations involving rhodopsin or analog pigments were carried out under dim red light ($\lambda > 620$ nm; Schott RG 620 cut-off filter).

Pigments were generated by incubating opsin membranes overnight with a 3 – 5-fold excess of 11*Z*-retinal or analog II (Scheme 1) at room temperature. For this, the membranes were either suspended in buffer A (20 mM Pipes, 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.1 mM EDTA, 1 mM dithioerythreitol, pH 6.5), or dissolved in 10 mM dodecyl maltoside in buffer A, to a final concentration in opsin of 20 – 30 µM. The retinals were added in a small volume of dimethylformamide (< 2% final volume) and the incubation was performed under an inert atmosphere (N_2 or argon). The kinetics of the regeneration were followed using a fivefold excess of retinals by taking spectra at successive time intervals on a PU 8800 spectrophotometer in the end-on photomultiplier setup to minimize losses due to light scattering. Rates were determined from semilog plots, using the 540-nm absorbance where the retinals do not absorb. Under these conditions, analog pigment formation was complete in 6 – 8 h. Regeneration was achieved most efficiently by adding three successive aliquots of retinal analog with a time interval of 4 – 6 h to a final threefold excess over opsin. The latter procedure was used routinely. The resulting analog membranes were recovered by centrifugation (30 min, 100000 × *g*, 4°C) and stored as a pellet at –80°C under an inert atmosphere. Subsequent incubation of analog pigments with 11*Z*-retinal usually produced less than 5% rhodopsin on the basis of the original amount of regeneratable opsin, indicating that 95 – 100% of the available opsin had reacted to produce an analog pigment.

Preparation of the analog pigment of fluoride III (Scheme 1) was performed as described before [16]. The resulting analog membrane, which still contained 60 – 70% opsin [16], was purified in the same way as analog pigments II except that 1 M NaCl had to be added to elute analog pigment III. The resulting pigment shows a maximal absorbance at 390 nm with an A_{280}/A_{390} ratio of 3.1 [16].

Purification and reconstitution of analog pigments and preparation of membrane films

Purification of analog pigments was accomplished by the same procedures as developed for rhodopsin (affinity chromatography over concanavalin-A – Sepharose at 4°C, followed by filtration through Sephadex G-25 [18, 20]). Analog membranes were dissolved in buffer B (20 mM nonyl glucoside in buffer A) by incubation for 30 min at 4°C under argon. The resulting solution was clarified by centrifugation (30 min, 100000 × *g*, 4°C) and applied (1 ml/h) to a concanavalin-A – Sepharose column (Pharmacia-LKB, Sweden; 1 ml bed volume/100 nmol pigment). The column was washed with 10 vol. buffer B to elute non-opsin protein, lipids and excess II. Since retinals are more easily eluted as the oxime [20], the first 3 vol. wash buffer contained 50 mM hydroxylamine to convert the excess II into the corresponding oxime. The analog pigment was then eluted with buffer B containing 200 mM methyl α-mannoside [20]. Fractions were screened by spectroscopic analysis and those containing analog pigment were combined, passed through a column of Sephadex G-25 (Pharmacia-LKB, Sweden) or polyacrylamide 10DG (Bio-Rad, Richmond, USA) to remove the methyl mannoside (3 bed vol./vol. pigment solution) and, if required, concentrated by centrifugation (Centricon-30 filters; Amicon Corp, Danvers, MA, USA) and stored at –80°C under argon.

Analog pigments were reconstituted into proteoliposomes by step-dilution of detergent in the presence of phospholipids as described [21]. Bovine brain phosphatidylethanolamine (Lipid Products, S. Nutfield, UK) was used as phospholipid, since it supports the photolytic integrity of rhodopsin equally as well as phosphatidylserine [21, 22] but is not prone to structural changes in the presence of bivalent cations. The molar ratio of phosphatidylethanolamine/analog pigment was kept at about 30:1 to avoid formation of non-bilayer structures. The resulting analog proteoliposomes were collected by centrifugation (30 min, 100000 × *g*, 4°C), washed once with buffer A or twice-distilled water and stored as a pellet at –80°C under argon.

The isopotential spin-dry procedure [23], as adapted for rod outer segment membranes and rhodopsin proteoliposomes [19], was used to deposit films of analog proteoliposomes on cellulose acetate coverslips. The coverslips were cleaned by repeated dipping in 95% ethanol and twice-distilled water and the analog proteoliposomes were suspended in twice-distilled water to a pigment concentration of about 10 nmol/ml, about 3 nmol/film pigment being deposited. The resulting membrane films are nearly transparent and very suitable for spectroscopic analysis. Before such analysis, the films were hydrated by applying a drop of buffer A and, since they rapidly dehydrate again when exposed to air, then placed in a sealed cuvet containing a small reservoir of twice-distilled water or buffer A.

Thermal stability

The relative thermal stability of the pigments was measured as described [24]. The decrease of absorbance in the visual region was taken as a measure of the thermal denaturation of the pigment. This approach yields single-order kinetics [24] and rates were calculated from semilog plots of the remaining 540-nm absorbance, where released retinals do not interfere, versus time.

Phosphodiesterase assay

Activation of cGMP phosphodiesterase through activated GTP-binding protein (G-protein) was used to assess whether the analog pigments could sustain phototransduction, i.e. could act as a functional photoreceptor. The hypotonic extract of isotonically washed rod outer segments [25, 26] served

Table 1. *Selected ¹H-NMR data of the four 10,20-methanoretinals (R6)*
The structures of the isomers and numbering of the C atoms are shown in Scheme 1. Spectra were taken in deuterated chloroform at 300 Hz (Bruker WM-300). Shifts are relative to tetramethylsilane. Figure between parentheses presents coupling constant for adjacent hydrogens. m = multiplet

R6 isomer	Chemical shift (coupling constant) of										
	H-7	H-8	H-11	H-12	H-14	H-15	H-20	H-20a	H-16	H-18	H-19
	ppm (Hz)										
7E,9E,13E	6.41	6.61	7.03	6.25	5.88	10.06	2.98	2.69	1.05	1.75	2.03
	(16.0)		(10.1)		(8.3)		(m)				
7E,9E,13Z	6.40	6.59	7.04	7.08	5.74	10.16	2.56	2.68	1.04	1.74	1.99
	(18.5)		(10.1)		(8.1)		(m)	(m)			
7E,9Z,13E	6.30	6.70	7.13	6.18	5.87	10.06	3.00	2.64	1.04	1.74	2.00
	(15.9)		(9.8)		(8.3)		(m)	(m)			
7E,9Z,13Z	6.29	6.71	7.16	7.03	5.74	10.15	2.61	2.61	1.03	1.73	1.98
	(15.9)		(10.1)		(8.2)		(m)				

as the source for G-protein and cGMP phosphodiesterase. Phosphodiesterase activity was assayed by monitoring proton release accompanying hydrolysis of cGMP to GMP at pH 7.9 [27]. A Schott N5900A combination micro pH electrode was used connected to a Radiometer PHM 82 pH-meter, of which the analog output signal was amplified and fed into a Kipp BD40 recorder.

Depleted rod outer segment membranes [25] or proteoliposomes of rhodopsin or analog pigment, containing 0.5–1 nmol pigment, were combined with about 20 µl hypotonic extract, which approximates the natural ratio. The mixture was brought to 200 µl by addition of buffer C (115 mM NaCl, 2 mM $MgCl_2$, 2.5 mM KCl, 0.1 mM NADPH, 10 mM Hepes pH 7.9). After addition of a concentrated GTP solution (1 mM final concentration) and a cGMP solution (2 mM final concentration), the 'dark' rate of phosphodiesterase activity was followed for 1 min. The mixture was then illuminated to trigger the maximal possible activation of the phosphodiesterase, usually requiring at least 0.01% rhodopsin to be bleached, and the light reaction was followed for at least 2 min. Finally, every recording was calibrated by adding known aliquots of HCl. The slopes of the curves were corrected for electrode drift and then used to calculate initial rates of dark and light reaction, which then were converted to hydrolysis rates of cGMP.

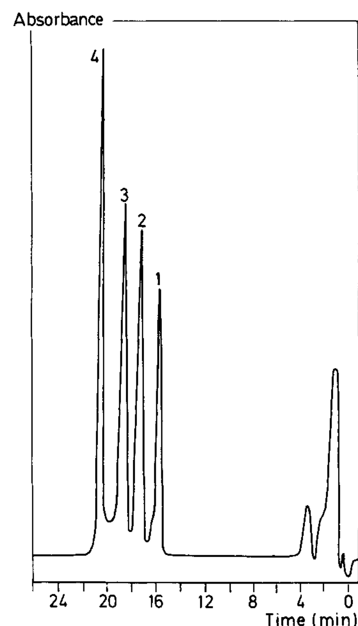


Fig. 1. *HPLC profile of the 10,20-methanoretinals obtained through Scheme 1.* Straight-phase separation (Lichrosorb Si-60) using isocratic elution with ether/petroleum ether (1/9, by vol.), 1.6 ml/min and detection at 370 nm. 1 = *7E,9Z,13Z*; 2 = *7E,9E,13Z*; 3 = *7E,9E,13E*; 4 = *7E,9Z,13E*

RESULTS

Characteristics of 10,20-methanoretinals (II)

The four geometric isomers of 10,20-methanoretinal (R6) obtained according to Scheme 1 could be easily resolved by HPLC (Fig. 1) and identified by ¹H-NMR (Table 1). The low coupling between H-11 and H-12 (≈ 10 Hz) demonstrates that all compounds have the 11*Z* geometry. NOE difference spectra were used to confirm the assignment of H-11/H-12 and H-20a/H-20 (irradiation of H-7/8) and subsequently to identify the compounds (irradiation of H-19 or H-14). This assignment agrees completely with the chemical shift pattern: the 9*Z* isomers show the 0.1-ppm downfield shift of H-8 relative to 9*E*, which, due to the presence of the 20a-CH_2 group, is much smaller than between (11*Z*)- and (9*Z*,11*Z*)-retinal (≈ 0.5 ppm [28, 29]). A much smaller shift (0.2 ppm) has also been reported for the five-membered ring 10,20-cycloretinal (R5) [6]. The (13*Z*)-isomers demonstrate a 0.8-ppm downfield shift of H-12, which is only about 0.2 ppm in retinal and 0.6 ppm in R5 but of comparable magnitude (0.8 ppm) in 10,20-ethanoretinal (R7) [5]. The (13*Z*)-isomers further show an 0.4-ppm downfield shift of H-20 (0.3 ppm in retinal and 0.25 ppm in R5). The data suggest that the structure of R6 resembles R7 more than R5. Nevertheless, the relatively high chemical shift of H-11 (≈ 7.1 vs ≈ 6.8 ppm) and the ≈ 9-nm redshift of the protonated Schiff base (Table 2) indicate that R6 has a higher coplanarity in its polyene system than (11*Z*)-retinal.

Table 2. *Absorbance data of the four 10,20-methanoretinals (R6) and various derivatives*
Presented are the individual retinals (AL), corresponding unprotonated (SB) and protonated (SBH^+) Schiff base with *n*-butylamine, and the corresponding rhodopsin analog (RH-A) with opsin shift (OS). For comparison, the same data are given for (11*Z*)-retinal, (9*Z*)-retinal and (9*Z*,11*Z*)-retinal [28, 29] (and R. S. H. Liu, personal communication). Solvent 1 = hexane, solvent 2 = methanol

Compound	λ_{max} (opsin shift) of derivative					
	AL		SB	SBH^+	RH-A	(OS)
	1	2	2	2		
	nm					(cm^{-1})
(11*Z*)-Retinal	365	375	350	440	498	(2650)
(9*Z*)-Retinal	363	372	349	440	485	(2110)
(9*Z*,11*Z*)-Retinal	352	367			472	(≈1800)
10,20-Methanoretinal:						
7E,9E,13E	374	392	368	449	510	(2660)
7E,9E,13Z	369	384	362	446		
7E,9Z,13E	366	386	360	447		
7E,9Z,13Z	360	377	354	451	494	(1930)

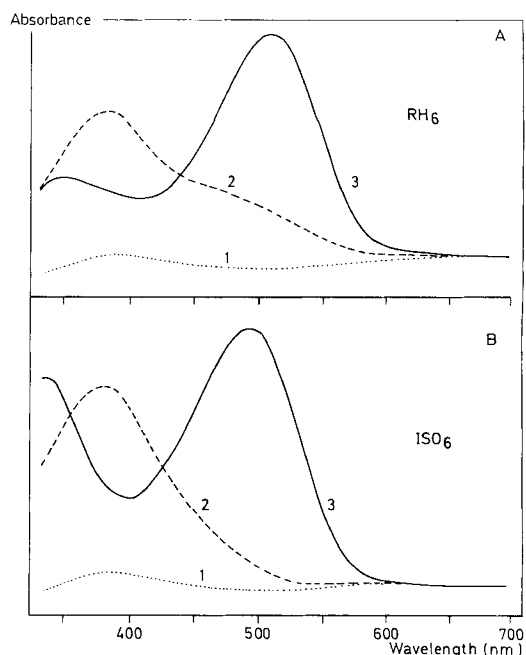


Fig. 2. *Analog pigment formation upon incubation of opsin membranes with a slight excess of either (*7E,9E,13E*)-10,20-methanoretinal (A) or (*7E,9Z,13E*)-10,20-methanoretinal (B).* The reference cuvet contained opsin membranes with only solvent (dimethylformamide) added. Spectrum 1 = base-line before addition; 2 = several minutes after addition of a slight excess of the retinal analog; 3 = after overnight incubation

Interaction of 10,20-methanoretinals with bovine opsin

Since bovine opsin retains its regeneration capacity in dodecyl maltoside [24], incubation of the (7*E*,9*E*,13*E*)-isomer of R6 with opsin was performed in membrane suspension as well as in dodecyl maltoside solution. A spectrally identical analog pigment was obtained under both conditions. The reaction rate was significantly higher in dodecyl maltoside solution, just as was observed for the regeneration with (11*Z*)-retinal. However, since regeneration in dodecylmaltose solution complicates subsequent purification and reconstitution, all further data have been obtained with analog pigments generated in membrane suspension. Some relevant results are compiled in Table 2.

Upon incubation with opsin membranes, (7*E*,9*E*,13*E*)-R6, the equivalent of (11*Z*)-retinal, yields an analog pigment with maximal absorbance at 510 nm (Fig. 2A). In order to test the stability of the pigment in detergent solution, we investigated three detergents, which vary strongly in 'destabilization potential' [24], each at a concentration of 20–30 mM. At room temperature the pigment was stable for at least 8 h in the very mild dodecyl maltoside, as well as in the intermediate type nonyl glucoside and the more aggressive Ammonyx-LO. The 9-*cis* isomer (7*E*,9*Z*,13*E*)-R6, the equivalent of (9*Z*,11*Z*)-retinal, showed comparable behaviour. Upon incubation with opsin membranes, it produces an analog pigment with maximal absorbance at 494 nm (Fig. 2B), which was also stable in the three detergents. The (13*Z*)-isomers yielded analog pigments which were spectrally exactly similar to the corresponding (13*E*)-isomer, i.e. (7*E*,9*E*,13*Z*)-R6 produced a 510-nm-absorbing pigment and (7*E*,9*Z*,13*Z*)-R6 produced a 494-nm-absorbing pigment. Since the C13 = C14 bond is prone to thermal isomerization [30], we assume that the (13*Z*)-isomer and the corresponding (13*E*)-isomer produce the same analog pigment due to thermal isomerization around the C13 = C14 double bond during incubation. Preliminary results, obtained via extraction of the chromophores as the retinal oximes [31] and analysis by HPLC, support this interpretation (De Grip, Van Oostrum and Van Groningen, unpublished). Hence we designate the 510-nm-absorbing pigment as Rh6, in analogy to Rh5 and Rh7, and the 494-nm-absorbing pigment as Iso6, since it derives from the (9*Z*)-isomer although it really represents a 9*Z*,11*Z*-rhodopsin analog rather than an isorhodopsin analog.

We compared the rate of pigment formation for the four isomers of R6 with that of (11*Z*)-retinal under the same experimental conditions of pseudo-first-order kinetics (fivefold excess of retinals) as described in Methods. The rates of the four retinal analogs were close. The highest rate was observed for (7*E*,9*E*,13*E*)-R6, the (11*Z*)-retinal equivalent, and was about 10% of that of (11*Z*)-retinal itself. The rate of the corresponding (13*Z*)-isomer, (7*E*,9*E*,13*Z*)-R6, was about 30% lower. Remarkably, the opposite was observed for the (9*Z*)-isomers. The rate of the (13*Z*)-isomer (7*E*,9*Z*,13*Z*)-R6 was close (85–90%) to that of (7*E*,9*E*,13*E*)-R6, while the rate of the (13*E*)-isomer, (7*E*,9*Z*,13*E*)-R6, was only 60–70% of the corresponding (13*Z*)-isomer. The rate of pigment formation with (7*E*,9*E*,13*E*)-III (the acyl fluoride of R6, cf. Scheme 1) was about 0.1% of that of (11*Z*)-retinal, i.e. about 100-fold slower than that of the corresponding aldehyde.

Thermal stability of the analog pigments

All analog pigments were stable in the presence of 50 mM hydroxylamine. Even in detergent solution (20 mM dodecyl maltoside or nonyl glucoside), no substantial decrease in maximal absorbance was observed after incubation for 24 h at room temperature with 50 mM hydroxylamine. In order to obtain an impression of the thermal stability of Rh6 relative to rhodopsin itself, we determined the temperature at which the half-life of the pigment is 10 min [24]. The value for Rh6

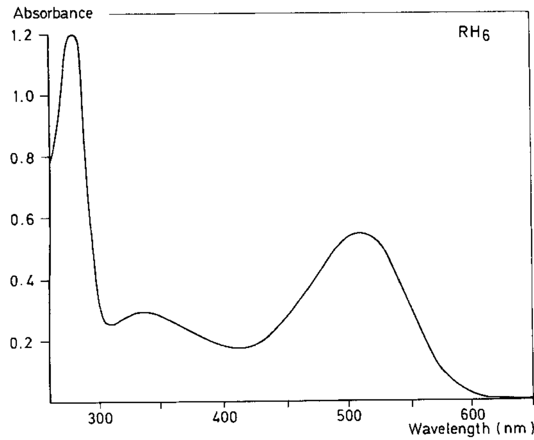


Fig. 3. *Spectrum of Rh6, (*7E,9E,*13*E*)-10,20-methanorhodopsin, after purification over ConA-Sepharose.* Detergent was 20 mM nonyl glucoside

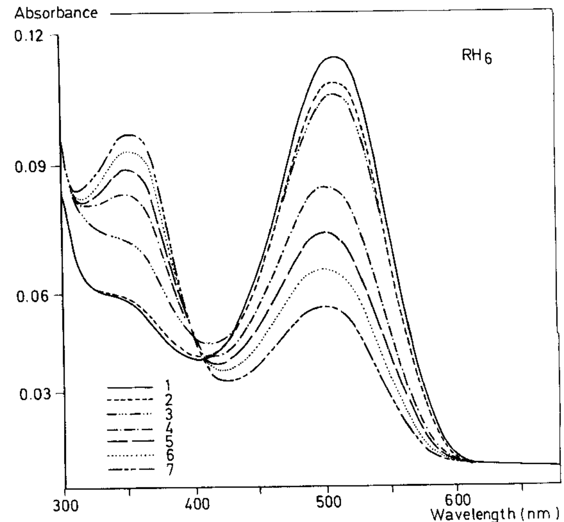


Fig. 4. *Photochemical behaviour of Rh6 in proteoliposomes with phosphatidylethanolamine (1/30 molar ratio), deposited as a membrane film on cellulose-acetate coverslips.* Spectrum 1, unilluminated Rh6 proteoliposomes after hydration of the membrane-film with buffer A; 2, following illumination with orange-red light (530-nm cut-off filter; intensity 700 lx); a small drop in the maximal absorbance is apparent but no further change occurs upon illumination under the same conditions during 6 min; 3, hydroxylamine was added to the reservoir (100 mM final concentration) and the solution brought into brief contact with the membrane film; 4–7, the film was then illuminated with white light (400–600 nm; intensity 3500 lx) for in total 10 min (4), 20 min (5), 30 min (6) and 45 min (7)

was only 4 ± 1 °C lower than that of rhodopsin itself (53 ± 1 °C [24]), which corresponds to just about a 5% decrease in the free energy of activation (ΔG^*; cf. [24]).

*Purification and reconstitution of (*7E,9E,*13*E*)-10,20-methanorhodopsin (Rh6)*

The analog pigments seemed sufficiently stable to attempt purification and reconstitution into artificial membranes. This and subsequent experiments were only performed with Rh6. Using affinity chromatography over ConA-Sepharose [18, 32], Rh6 could be completely purified from contaminant protein, lipid and excess R6. Recovery of Rh6 varied between 90–95%. The first three fractions containing Rh6 were combined (80–90% of the amount applied) and gel-filtered through 3 bed vol. Sephadex G-25 to remove the mannoside. A typical spectrum of Rh6 purified in this way is shown in Fig. 3. The λ_{max} remains stable at 510 nm during purification. The A_{280}/A_{510} ratio varied between 2.0–2.2 for different preparations. Since purified rhodopsin has a A_{280}/A_{500} ratio of 1.7–1.8, we estimate the molar absorbance coefficient of Rh6 at 34000 ± 2000 M^{-1} cm^{-1}. Purified Rh6 could be stored at −80 °C under an inert atmosphere for at least a year.

Reconstitution of Rh6 with phosphatidylethanolamine (30:1 molar ratio to Rh6) to prepare proteoliposomes was accomplished by the detergent step-dilution procedure [21]. The Rh6 proteoliposomes were recovered by centrifugation and used to prepare membrane films for spectroscopic analysis. The resulting films retained the Rh6 absorbance band with λ_{max} at 510 nm without any evidence of release of retinal (Fig. 4, spectrum 1), demonstrating that the analog pigment also withstood reconstitution and film deposition.

Photochemistry of Rh6 and Iso6

Preliminary studies on membrane suspensions indicated that under illumination conditions, which extensively bleached rhodopsin, little effect on the Rh6 spectrum was detectable. This was therefore investigated in more detail on membrane films (Figs 4, 5).

Illumination of membrane films of Rh6 proteoliposomes (Fig. 4, spectrum 1) with orange light, showed a very rapid response (within 10 s), consisting of a slight decrease in maximal absorbance (spectrum 2). Upon further illumination under the same conditions for up to 6 min, no further change was observed. The spectra taken in this time interval were identical to spectrum 2. Under the same conditions, a film containing rhodopsin is over 90% bleached. We checked whether any metastable photointermediates were generated in Rh6 after this 6-min illumination by briefly immersing the film into buffer A containing 100 mM hydroxylamine. This led to only a small decrease in maximal absorbance (spectrum 3). In darkness no further effect of hydroxylamine was observed and spectrum 3 was stable. The stability of Rh6 in the light in the presence of hydroxylamine was then tested by increasing the light intensity fivefold and prolonging the incubation time. Upon subsequent 10-min exposures, yielding spectra 4–7, the main absorbance band slowly decreased while an absorbance peak at 362 nm, representing the retinal-oxime, emerged. In addition, the maximal absorbance of the main band slowly shifted from 510 nm to 500 nm. To investigate whether the release of retinal and shift of λ_{max} under continuous illumination was either inherent to Rh6 or induced by hydroxylamine, the experiment was repeated on films of Rh6 membranes obtained directly after regeneration, without addition of hydroxylamine (Fig. 5A). Again the rapid drop in maximal absorbance upon short light exposure is apparent (Fig. 5A, spectrum 5). Longer exposures induce a slow decrease in maximal absorbance and a shift of the λ_{max} finally to 498 nm. This shift is comparable to that seen in Fig. 4, but the decrease in main-band absorbance is much less pronounced than in the presence of hydroxylamine (cf. spectrum 5 of Fig. 4 with spectrum 20 of Fig. 5).

The same experiment was performed on membrane films containing Iso6. Again a slow decrease in main-band absor-

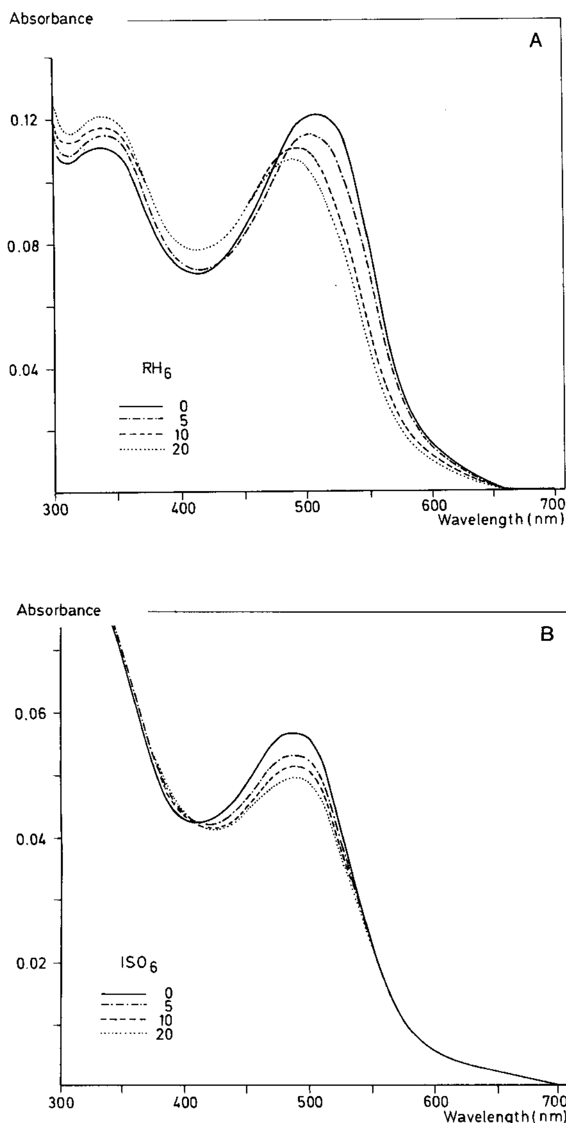


Fig. 5. *Photochemical behaviour of Rh6 (A) or Iso6 (B) membranes, obtained after regeneration of opsin membranes with (*7E,9E,*13*E*)-R6 or (*7E,9Z,*13Z)-R6, deposited as a membrane film on cellulose-acetate coverslips.* Spectra were taken after 0, 5, 10 and 20 min of illumination (white light; 3500 lx) as indicated. The residual peak around 350 nm and the stronger interference from light scattering are due to the presence of excess R6. When corrected for light scattering the λ_{max} in A shifts from 510 nm to 507 nm after a 5-min exposure, to 500 nm after a 10-min exposure and to 498 nm after a 20-min exposure. In B the λ_{max} shifts to about 496 nm after 10 min and to about 498 after 20 min of illumination

bance was noted, but now a slow red-shift in maximal absorbance from 494 nm to 498 nm was observed (Fig. 5 B). Apparently, whether starting from the (7*E*,9*E*,13*E*)- or (7*E*,9*Z*,13*Z*)-analog pigment, continuous illumination slowly produces the same steady state.

Finally, the photostability of Rh6 in detergent solution (20 mM dodecyl maltoside in buffer A) was compared to that of rhodopsin. Under conditions of relatively low light intensity where rhodopsin decayed with a half-time of 60 ± 2 s, Rh6 decayed with a half-time of 160 ± 1 min, i.e. about 160-fold slower than rhodopsin.

Table 3. *Light-triggered activation of rod cGMP phosphodiesterase* Activation is measured for rhodopsin membranes (Rh-M), or proteoliposomes containing either rhodopsin (Rh-P), (7*E*,9*E*,13*E*)-10,20 methanorhodopsin (Rh6-P) or (7*E*,9*E*,13*E*)-10,20 methanoretinoyl opsin (RhIII-P). The activity in the light is the maximal one obtained. This requires quite different light intensities for the different preparations. A relative figure for the required intensity (*I*), with Rh-M normalized to 1, is shown between parentheses. Values are means ± SEM with number of determinations (*n*) between parentheses in the last column

Activating system	Phosphodiesterase activity in		Activation
	dark	light (*I*)	
	nmol cGMP hydrolyzed/min		%
Rh-M	44 ± 11	201 ± 52 (1)	341 ± 25 (*n* = 9)
Rh-P	26 ± 3	103 ± 8 (10)	304 ± 9 (*n* = 5)
Rh6-P	12.5 ± 4.5	16.9 ± 4.8 (> 100)	40 ± 22 (*n* = 3)
RhIII-P	9.2 ± 1.2	9.2 ± 1.2 (> 1000)	0 (*n* = 3)

Signal expression by Rh6

In order to evaluate whether Rh6 would be able to sustain phototransduction, proteoliposomes were combined with a hypotonic extract from bovine rod outer segments, containing the required transductory elements like G-protein and cGMP phosphodiesterase, such that about the natural ratio between these proteins and rhodopsin was restored. After addition of GTP and cGMP, the dark activity of phosphodiesterase was measured and subsequently the light-triggered activity was monitored at various light intensities. Under these conditions rhodopsin membranes maximally activate phosphodiesterase by at least 300% (Table 3) already at very low light intensities (about 0.01% bleached). The dark and light activity can vary considerably (3–4-fold) between hypotonic extracts, but the degree of activation is fairly consistent (Table 3). However, this 300% activation is 2–4-fold lower than that measured in isolated intact rod outer segments under identical conditions (De Grip et al., unpublished), probably due to the much higher dilution of the transductory components in the recombinant system. Rhodopsin proteoliposomes show a reduction in both dark and maximal light activity, but the degree of maximal activation is not significantly reduced (Table 3). Maximal activation is, however, only achieved at about 10-fold higher bleaching levels ($\approx 0.1\%$). This probably reflects the presence of multilamellar liposomes and the symmetric reconstitution of rhodopsin into the artificial membranes. Proteoliposomes with (7*E*,9*E*,13*E*)-10,20-methanoretinoyl opsin (RhIII) produce no detectable light activation of phosphodiesterase whatsoever, even at light intensities which would bleach 30–40% rhodopsin (Table 3). Proteoliposomes with Rh6 are not completely inactive. High light intensities are, however, required to produce rather modest and rather variable activation (20–50%).

DISCUSSION

*Six-ring 11-*cis*-locked retinals generate rhodopsin-similar analog pigments Rh6 and Iso6*

Starting from β-ionone, four stereoisomers of 10,20-methanoretinal (R6; compound II in Scheme 1) could be

conveniently prepared in about 15% overall yield according to Scheme 1 and [16]. This synthetic route affords a mixture of the (7*E*,9*E*,13*E*)-, (7*E*,9*E*,13*Z*)-, (7*E*,9*Z*,13*E*)- and the (7*E*,9*Z*,13*Z*)-isomers, which could be base-line separated and purified by isocratic HPLC. The individual peaks could be assigned unambiguously using ^{1}H-NMR in combination with NOE analysis. The NMR data and the red-shift of the visible absorbance bands indicate that the presence of the ring induces higher coplanarity in the polyene chain of R6 than in (11*Z*)-retinal itself, but less extreme than in the case of the five-membered analog R5 [6].

A smooth reaction of all four isomers with bovine opsin was observed at a relatively high rate: 5–10% of that of (11*Z*)-retinal. Incubation of opsin with either of the two (9*E*)-isomers led to the same analog pigment with maximal absorbance at 510 nm (Rh6). The same is the true for the two (9*Z*)-isomers, yielding an analog pigment with maximal absorbance at 494 nm (Iso6). The rate of pigment formation decreased in the order 7*E*,9*E*,13*E* > 7*E*,9*Z*,13*Z* > 7*E*,9*E*,13*Z* > 7*E*,9*Z*,13*E*. It is well documented that the C13 = C14 double bond of retinal undergoes thermal isomerization relatively easily, in particular upon Schiff base formation with amino-group-containing compounds [30, 33]. Indeed, incubation of opsin with (9*Z*,13*Z*)-retinal leads almost exclusively to formation of the (9*Z*)-pigment (isorhodopsin) unless the reaction is carried out in digitonin solution with 3–5-fold excess of opsin [34]. Likewise, the (13*E*)- and (13*Z*)-congeners of R7 yield pigments with very similar properties [5]. Hence, we conclude that the pigments obtained under our reaction conditions (excess retinal, membrane suspension) have the same chromophore structure, whether derived from the (13*Z*)- or the (13*E*)-isomer, due to thermal isomerization around this bond during incubation prior to reaction with opsin.

The correct chromophore structure may be derived from kinetic data. When thermal isomerization is required to generate the correct chromophore, the rate of formation of analog pigment will be slowed down. From the kinetic data for the four isomers of R6, we therefore conclude that the chromophore structure in the 510-nm-absorbing pigment is 7*E*,9*E*,13*E* and that in the 494-nm-absorbing pigment is 7*E*,9*Z*,13*Z*. This agrees with the dimensional preference of the chromophore binding site [35], as also schematically shown in Scheme 1: the molecular structure of the (7*E*,9*E*,13*E*)- and the (7*E*,9*Z*,13*Z*)-congeners is very similar. Direct experimental proof for this conclusion requires extraction of the chromophore [31] and we are presently developing a reliable system to separate and analyse the oxime derivatives of R6. Preliminary results support the assignment given above.

The following properties strongly suggest that the binding site of opsin, and the overall protein structure, are very little perturbed upon occupation with R6, i.e. that the chromophore structure in Rh6 is very similar to that in rhodopsin itself. (a) The reaction rate, 5–10% of that of (11*Z*)-retinal, is relatively high. For comparison, the formation rate of Rh5 from R5 is only 0.15% of that of rhodopsin [7]. (b) The opsin shift, i.e. the red-shift in absorbance maximum of the analog pigment relative to the protonated Schiff base of the retinal analog (e.g. [2]), is nearly identical for rhodopsin (2650 cm^{-1}) and Rh6 (2660 cm^{-1}). Likewise, the opsin shift of Iso6 (1930 cm^{-1}) is in between that of isorhodopsin (2110 cm^{-1}) and (9*Z*,11*Z*)-rhodopsin ($\approx$ 1800 cm^{-1}). Analog pigments of (9*Z*,11*Z*,13*Z*)-retinal have not yet been reported. (c) At room temperature Rh6 and Iso6 are as stable as rhodopsin towards hydroxylamine (tested up to 50 mM) and towards solubilization by the detergents dodecyl β-1-maltoside, nonyl β-1-glucoside and Ammonyx-LO. (d) The thermal stability of Rh6 in 20 mM nonyl β-1-glucoside solution at pH 6.5 is only slightly less than that of rhodopsin, corresponding to merely a 5% decrease in activation energy. Hence, it appears that among the three hitherto prepared 11-*cis*-locked rhodopsin analogs Rh5 [6, 7], Rh6 and Rh7 [5], Rh6 is structurally most similar to rhodopsin.

As is expected for an 11-*cis*-locked analog [5–8], Rh6 is much more photostable than rhodopsin. Nevertheless, it does not appear to be completely photostable. In detergent solution Rh6 bleaches with about 0.6% of the rate of rhodopsin. This phenomenon will be discussed in more detail below. Extreme photostability can only be achieved by an additional modification: substitution of the Schiff base link between chromophore and opsin for an amide link, as in the analog pigment RhIII derived from opsin and analog III (Scheme 1), the acid fluoride derivative of R6 [16]. RhIII also displays considerably higher thermal stability than rhodopsin [16]. Since the amide configuration is substantially less sensitive to hydrolytic cleavage than the Schiff base, hydrolysis of the Schiff base is probably one of the factors which determine the photostability of Rh6 and the thermal stability of Rh6 and rhodopsin.

Photochemistry of Rh6 and Iso6

The 11-*cis* lock in Rh5 and Rh7 precludes the light-induced formation of the normal photointermediates [5–9]. Instead, illumination of Rh5 at 0 °C establishes a photo-mixture of the parent 495-nm-absorbing pigment and a 466-nm-absorbing one, which probably has a different side-chain orientation due to twists around single bonds [7]. Rh6 behaves differently. Normal photointermediates are indeed probably not produced. Analysis of Rh6 by Fourier-transform infrared spectroscopy reveals no light-induced changes whatsoever at 83 K or 130 K, where bathorhodopsin is stable (De Grip and Rothschild, unpublished). At room temperature however, definite changes in protein and chromophore vibrations are observed, but of much smaller size (< 10%) than common to the rhodopsin → metarhodopsin II transition (e.g. [19]). These changes probably represent the structural counterpart of the absorbance changes shown in Figs 4 and 5. Continuous illumination of Rh6 or Iso6 rapidly leads to a small ($\approx$ 5%) loss in absorbance followed by a very slow further decrease around 500 nm and increase between 350–400 nm. The latter process is strongly accelerated in the presence of hydroxylamine. Remarkably, the initial stage of this latter slow process is accompanied by a slow shift in maximal absorbance, such that the same final species absorbing at 498 nm is reached, irrespective of whether the starting point was Rh6 (510 nm) or Iso6 (494 nm). Furthermore, the bandwidth of the main absorbance band does not change significantly during the shift in absorbance maximum, suggesting that a photo-mixture of two or more species is not involved. The present evidence suggests that the continuous energy input into the chromophore during illumination allows the selection of a structure which would be unfavorable in free retinal but which fits the binding site better than the parent (7*E*,9*E*,13*E*)- or (7*E*,9*Z*,13*Z*)-R6. This probably involves isomerization and/or twist around the C9 = C10 double bond to account for the red shift of Iso6 and a twist around single bonds to account for the blue shift of Rh6. In addition, during the photochemically induced transition(s), the Schiff base link is apparently somewhat more exposed and becomes transiently more accessible to hydrolytic cleavage, resulting in a slow rate of hydrolysis and loss of pigment. This would explain the observation that the latter process is

substantially accelerated by the action of hydroxylamine, which is a much stronger nucleophile than H_2O.

Physiological activity of Rh6

Very low bleaching levels (0.01 – 0.1%) already maximally activate the physiological response of rhodopsin (binding and activation of G-protein followed by activation of phosphodiesterase [25, 27]) even in recombined systems (cf. Table 3). In spite of the fact that $11Z \rightarrow$ all-E isomerization is precluded, Rh6 shows some physiological activity but at a much reduced level (up to 15% of rhodopsin) and requiring higher light intensities (Table 3). This remnant activity probably originates in the structural changes, produced during the complex photochemical transitions, which also trigger the absorbance shifts and slow release of retinal by hydrolysis (Figs 4 and 5) and which probably are of a much more stochastic nature than the vectorial changes accompanying the rhodopsin $\rightarrow$ metarhodopsin II transition. This concept is supported by the observation that the analog pigment RhIII, which cannot undergo hydrolytic release of its chromophore, has no detectable physiological activity (Table 3). Hence, we conclude that the old dogma, first formulated by George Wald (e.g. [36]) and stating that isomerization of 11*Z* to all-*E* is essential for normal physiological activity of rhodopsin, is fully supported by the behaviour of Rh6. This further agrees with elegant *in vivo* experiments using R7 [13]: supplementation of vitamin-A-depleted rats with R7 led to the formation of analog pigment Rh7 in ocular tissue. However, this did not increase *b*-wave sensitivity and rather suppressed the sensitizing action of a subsequent dose of vitamin A [13], a strong indication that Rh7 is not physiologically active. On the other hand, it has recently been reported that supplementation of a blind mutant of *Chlamydonomas reinhardtii* with R7 very effectively restored its phototaxis [11, 12]. This was taken as solid evidence that phototransduction does not require 11*Z* to all-*E* isomerization. However, the same studies show that administration of (all-*E*)-retinoyl fluoride restored phototaxis also and almost equally effectively as (11*Z*)-retinal [11, 12]. Previously, we have demonstrated that (all-*E*)-retinoyl fluoride does not react with bovine opsin to produce an analog pigment [16]. In addition, the 11-*cis*-locked fluoride III reluctantly forms a rhodopsin analog pigment, but the latter does not show any physiological activity (Table 3). Hence, we have to conclude that the vertebrate visual pigments and the *Chlamydonomas* phototaxis operate by different mechanisms and the results obtained in one system should not be extrapolated to the other.

Conclusion

The rhodopsin analog pigments generated with the 11-*cis*-locked retinals (7*E*,9*E*,13*E*)-10,20-methanoretinal (Rh6: 510-nm absorbing) and (7*E*,9*Z*,13*Z*)-10,20-methanoretinal (Iso6: 494-nm absorbing) are highly akin to rhodopsin with respect to optical properties and thermal stability. However, they are much more photostable and only express some physiological activity at higher light intensities. These compounds should therefore be very suitable as a control for functional studies on rhodopsin and as an alternative to rhodopsin in studies exploring properties which do not depend on light-triggered $11Z \rightarrow$ all-E isomerization of its chromophore.

We thank Mrs W.H.M. van Groningen-Luyben for performing the phosphodiesterase assays. These investigations were in part financially supported by the Netherlands Organization for Scientific Research (N.W.O.) through the subdivisions Space Research Organization of the Netherlands (SRON MG-001, to WdG) and the Netherlands Foundation for Chemical Research (SON, to WdG and to JL).

REFERENCES

1. Kropf, A., Whittenberger, B. P., Waggoner, A. & Goff, S. P. (1973) *Exp. Eye Res. 17*, 591–606.
2. Derguini, F. & Nakanishi, K. (1986) *Photobiochem. Photobiophys. 13*, 259–283.
3. Crouch, R. K. (1986) *Photochem. Photobiol. 44*, 803–807.
4. Liu, R. S. H., Asato, A. E., Denny, M., Mead, D., Mirzadegan, T. & Zhang, B.-W. (1988) in *Proceedings of Yamada Conference XXI* (T. Hara, ed.) pp. 43–48, Yamada Science Foundation, Osaka.
5. Akita, H., Tanis, S. P., Adams, M., Balogh-Nair, V. & Nakanishi, K. (1980) *J. Am. Chem. Soc. 102*, 6370–6372.
6. Ito, M., Kodama, A., Tsukida, K., Fukada, Y., Shichida, Y. & Yoshizawa, T. (1982) *Chem. Pharm. Bull. 30*, 1913–1916.
7. Fukada, Y., Shichida, Y., Yoshizawa, T., Ito, M., Kodama, A. & Tsukida, K. (1984) *Biochemistry 23*, 5826–5832.
8. Mao, B., Tsuda, M., Ebrey, T. G., Akita, H., Balogh-Nair, V. & Nakanishi, K. (1981) *Biophys. J. 35*, 543–546.
9. Buchert, J., Stefancic, V., Doukas, A. G., Alfano, R. R., Callender, R. H., Pande, J., Akita, H., Balogh-Nair, V. & Nakanishi, K. (1983) *Biophys. J. 43*, 279–283.
10. Birge, R. R., Murray, L. P., Pierce, B. M., Akita, H., Balogh-Nair, V., Findsen, L. A. & Nakanishi, K. (1985) *Proc. Natl Acad. Sci. USA 82*, 4117–4121.
11. Foster, K. W., Saranak, J., Derguini, F., Rao, V. J., Zarrilli, G. R., Okabe, M., Fang, J.-M., Shimizu, N. & Nakanishi, K. (1988) *J. Am. Chem. Soc. 110*, 6588–6589.
12. Foster, K. W., Saranak, J., Derguini, F., Zarrilli, G. R., Johnson, R., Okabe, M. & Nakanishi, K. (1989) *Biochemistry 28*, 519–524.
13. Crouch, R., Nodes, B. R., Perlman, J. I., Pepperberg, D., Akita, H. & Nakanishi, K. (1984) *Invest. Ophthalmol. Vis. Sci. 25*, 419–428.
14. Bonting, S. L., De Grip, W. J. & Daemen, F. J. M. (1984) in *Proceedings of the Workshop 'Protein single crystal growth under microgravity', ESA SP-1067* (Guyenne, T. D. & Hunt, J. J., eds) pp. 9–13, ESA Sci. Techn. Pub., Noordwijk.
15. Waddell, W. H., Lecomte, J., West, J. L. & Younes, V. E. (1984) *Photochem. Photobiol. 39*, 213–219.
16. Van der Steen, R., Groesbeek, M., Van Amsterdam, L. J. P., Lugtenburg, J., Van Oostrum, J. & De Grip, W. J. (1989) *Recl. Trav. Chim. Pays Bas 108*, 20–27.
17. De Grip, W. J. & Bovee-Geurts, P. H. M. (1979) *Chem. Phys. Lipids 23*, 321–335.
18. De Grip, W. J., Daemen, F. J. M. & Bonting, S. L. (1980) *Methods Enzymol. 67*, 301–320.
19. De Grip, W. J., Gillespie, J. & Rothschild, K. J. (1985) *Biochim. Biophys. Acta 809*, 97–106.
20. De Grip, W. J. (1982) *Methods Enzymol. 81*, 197–207.
21. De Grip, W. J., Olive, J. & Bovee-Geurts, P. H. M. (1983) *Biochim. Biophys. Acta 734*, 168–179.
22. Van Breugel, P. J. G. M., Geurts, P. H. M., Daemen, F. J. M. & Bonting, S. L. (1978) *Biochim. Biophys. Acta 509*, 136–147.
23. Clark, N. A., Rothschild, K. J., Luippold, D. & Simon, B. (1980) *Biophys. J. 31*, 65–96.
24. De Grip, W. J. (1982) *Methods Enzymol. 81*, 256–265.
25. Kühn, H. (1984) *Prog. Retinal Res. 3*, 123–156.
26. Miller, J. L., Litman, B. J. & Dratz, E. A. (1987) *Biochim. Biophys. Acta 898*, 81–89.
27. Liebman, P. A. & Evanczuk, A. T. (1982) *Methods Enzymol. 81*, 532–542.
28. Patel, D. (1969) *Nature 221*, 825–828.
29. Liu, R. S. H. & Asato, A. E. (1982) *Methods Enzymol. 88*, 506–516.

30. Groenendijk, G. W. T., Jacobs, C. W. M., Bonting, S. L. & Daemen, F. J. M. (1980) *Eur. J. Biochem. 106*, 119–128.
31. Groenendijk, G. W. T., De Grip, W. J. & Daemen, F. J. M. (1980) *Biochim. Biophys. Acta 617*, 430–438.
32. Steineman, A. & Stryer, L. (1973) *Biochemistry 12*, 1499–1502.
33. Bernstein, P. S., Fulton, B. S. & Rando, R. R. (1986) *Biochemistry 25*, 3370–3377.
34. Shichida, Y., Nakamura, K., Yoshizawa, T., Treman, A., Denny, M. & Liu, R. S. H. (1989) *Biochemistry 27*, 6495–6499.
35. Liu, R. S. H. & Mirzadegan, T. (1988) *J. Am. Chem. Soc. 110*, 8617–8623.
36. Wald, G. (1968) *Nature 219*, 800–807.