Effect of Variation of Retinal Polyene Side-Chain Length on Formation and Function of Bacteriorhodopsin Analogue Pigments†

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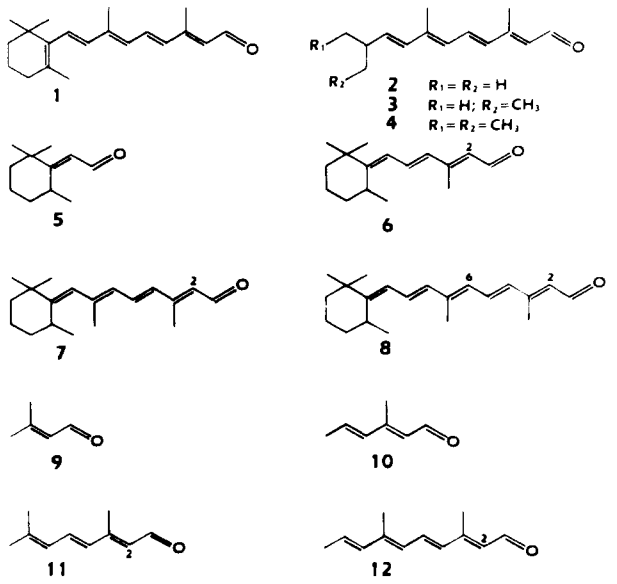
ABSTRACT: The effect of the length of the retinal polyene side chain on bacterioopsin pigment formation and function has been investigated with two series of synthetic retinal analogues. Cyclohexyl derivatives with polyene chains one carbon longer and one or more carbons shorter than retinal and linear polyenes with no ring have been synthesized and characterized. Compounds of six carbons or less in the polyene chain form pigments very poorly or not at all with bacterioopsin. Compounds containing at least seven carbons in the chain are found to form reasonably stable bacterioopsin pigments that show a small shift in absorbance on irradiation. However, photocycling and proton photorelease are not detected. The analogue with nine carbons in the polyene chain (one less than retinal) forms a stable pigment with an M-type intermediate but demonstrates reduced amounts of photocycling and light-activated proton release. The analogue with a polyene chain identical with that of retinal, but containing no ring, forms a pigment that shows both an efficient light-activated proton photocycle and release. The pigment containing the chromophore with the polyene chain one carbon longer than retinal is likewise fully active. We thus conclude that the length of the polyene chain must be at least 9 carbons for the formation of a stable pigment that photocycles and must be 10 carbons for both the photocycle and light-activated proton release to have a high quantum efficiency.

Bacteriorhodopsin is the only protein in the purple membrane of *Halobacterium halobium*. Upon the absorption of light, it undergoes a photocycle establishing a proton gradient across the membrane [reviewed in Stoeckenius et al. (1979) and Ebrey (1982)]. The chromophore of light-adapted bacteriorhodopsin is *all-trans*-retinal; its role in the photoactivated proton release and uptake, while obviously important, remains unclear.

The chromophore binding site of bacteriorhodopsin is specific for the all-trans and 13-cis isomers of retinal (**1**)



(Oesterhelt et al., 1973) and, to a lesser extent, the 7-cis isomer (Kohl & Sperling, 1979). We have previously shown that the cyclohexyl ring of retinal is not required for pigment formation, as acyclic retinals such as **2–4** can form stable pigments with bacterioopsin (Crouch et al., 1984). These results are in agreement with experiments showing that bacterioopsin does not have a specific cyclohexyl ring binding site (Towner et al., 1981). In addition, some of these acyclic pigments undergo photocycling and show light-activated proton release with an efficiency similar to that of native bacteriorhodopsin. The nonspecificity of the ring binding site is further demonstrated by the documentation of the large number of analogues that have extensive modification in the cyclohexyl ring and can still form stable, photoactive pigments with bacterioopsin. Several of these modified pigments have been shown to show light-activated proton release, e.g., naphthylretinal (Iwasa et al., 1984) and tetrazolylretinal, (Derguini et al., 1984).

Apart from the cyclohexyl ring of the retinal, the contribution of other structural features of retinal to the function of the pigment have not been systematically addressed. In this paper, we report the synthesis of retinals varying in polyene side-chain length. Compounds several carbons shorter (**5** and **6**), one carbon shorter (**7**), and one carbon longer (**8**) in the side chain have been prepared as well as a series containing acyclic polyene chains (**9–12**). In all these compounds the relationship of the polyene methyl groups to the aldehyde function is the same as in retinal itself. We describe the results of pigment regeneration with these retinals and report here the dependence of the photoactivated proton pumping on the length of the polyene chain.

Materials and Methods

All work on retinals was carried out under dim red light and under nitrogen. The retinals were stored at −70 °C under argon. Absorption spectra were recorded on a Varian Cary

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2200 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained on a Varian EM-390 and a Bruker WH-400 at the National Science Foundation facility at the University of South Carolina (CHE 78-18723). Mass spectra were obtained on a Finnegan 3200 with a direct probe using electron impact.

Chemicals. Diisobutylaluminum (DIBAL), activated manganese dioxide (MnO_2), pyridinium chlorochromate (PCC), methylmagnesium chloride, vinylmagnesium bromide, 3-methyl-2-buten-1-ol, and triethyl phosphonoacetate (C_2 phosphonate) were purchased from Aldrich Chemicals. Ethyl 4-(diethylphosphono)-3-methyl-2-butenoate (C_5 phosphonate) was prepared following published procedures [see, e.g., Mayer & Isler (1971)]. 2,2,6-Trimethylcyclohexanone was obtained from Pfaltz & Bauer, Inc., Stamford, CT. All other chemicals were reagent grade.

Synthesis of Retinal Analogues. The synthetic retinal analogue isomers were purified either by flash column chromatography (Still et al., 1978) using 10% ether/hexanes or by high-performance liquid chromatography (HPLC) using 2–5% ether/hexanes as solvent on a μPorasil column. All isomers were characterized by NMR (Table I), UV–vis (Table II), and mass spectroscopy.

(*a*) *6-(2′,2′,6′-Trimethylcyclohexylidene)-3-methyl-2,4-hexadienal* (**6**). 2,2,6-Trimethylcyclohexanone was reacted overnight with vinylmagnesium bromide at room temperature under N_2 with dry tetrahydrofuran (THF) solvent. The resulting 1-vinyl-2,2,6-trimethylcyclohexanol was oxidized to 2-(2′,2′,6′-trimethylcyclohexylidene)-2-ethanal (**5**) by using PCC in the presence of sodium acetate buffer (Dauben & Michno, 1977). Compound **5** was then coupled with triethyl phosphonoacetate in a standard Emmons type chain extension reaction to afford an ester that was reduced to the alcohol with DIBAL. The alcohol was finally oxidized to compound **6** with activated MnO_2 (Fatiadi, 1976) with an overall yield of 54%. Both the all-trans and 2-cis isomers of **6** were isolated (mass spectrum, *m/e* 232).

(*b*) *8-(2′,2′,6′-Trimethylcyclohexylidene)-3,7-dimethyl-2,4,6-octatrienal* (**7**). A THF solution of **5** and methylmagnesium chloride was allowed to stir overnight at room temperature under N_2. The resulting allylic alcohol was oxidized to 3-(2′,2′,6′-trimethylcyclohexylidene)-propan-2-one using MnO_2 which was condensed with triethyl phosphonoacetate to give ethyl 4-(2′,2′,6′-trimethylcyclohexylidene)-3-methyl-2-butenoate. The dienoate was successively reduced (DIBAL), oxidized (MnO_2), reacted with C_5 phosphonate, reduced, and finally oxidized to give compound **7** with 32% overall yield (mass spectrum, *m/e* 272, (M − CH_3) 257].

(*c*) *10-(2′,2′,6′-Trimethylcyclohexylidene)-3,7-dimethyl-2,4,6,8-decatetraenal* (**8**). The conventional chain-extension reaction between 6-(2′,2′,6′-trimethylcyclohexylidene)-3-methyl-2,4-hexadienal (**6**) and the C_5 phosphonate followed by the standard DIBAL reduction and MnO_2 oxidation afforded both the all-trans and 2-cis isomers of **8** in 51% overall yield. The 6-cis isomer of **8** was synthesized similarly starting with the 2-cis isomer of hexadienal **6** [mass spectrum, *m/e* 298, (M − CH_3) 283].

(*d*) *3-Methyl-2-butenal* (**9**) (*Fischer et al., 1931*). A mixture of 3-methyl-2-buten-1-ol and a 10-fold excess of MnO_2 in dichloromethane was stirred at room temperature for 5 h. The MnO_2 was then removed by filtration with the aid of Celite to give a 68% yield of compound **9**.

(*e*) *3-Methyl-2,4-hexadienal* (**10**) (*Duperrier et al., 1975*). Acetaldehyde was added to the sodium anion of C_5 phosphonate in dry THF and the reaction mixture stirred at 0 °C for 5 h to produce ethyl 3-methyl-2,4-hexadienoate which itself was reduced and then oxidized to compound **10** in 48% overall yield (mass spectrum, *m/e* 110).

(*f*) *3,7-Dimethyl-2,4,6-octatrienal* (**11**) (*Young & Linden, 1947*). The reaction of 3-methyl-2-butenal (**9**) and the C_5 phosphonate afforded ethyl 3,7-dimethyl-2,4,6-octatrienoate which was converted to compound **11** following the usual reduction/oxidation procedure. Overall yield was 47% [mass spectrum, *m/e* 150, (M − CH_3) 135].

(*g*) *3,7-Dimethyl-2,4,6,8-decatetraenal* (**12**). Ethyl 3,7-dimethyl-2,4,6,8-decatetraenoate, obtained by the condensation reactions of the C_5 phosphonate and 3-methyl-2,4-hexadienal (**10**), was transformed to compound **12** by using DIBAL reduction followed by MnO_2 oxidation. The overall yield was 54% [mass spectrum, *m/e* 176, (M − CH_3) 161].

Pigments. (*a*) *Regeneration.* Purple membrane was purified from cultures of *Halobacterium halobium* by the method of Becher and Cassim (1975). The method of preparing bleached membrane was modified from Tokunaga et al. (1977). Purple membrane was bleached by irradiation at 40 °C in a solution of 1 M NH_2OH, 0.1 M NaCl, and 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 9.5) for 4 h. The bleached membrane was washed 3 times in distilled water, then lyophilized, washed with hexane 5–6 times to remove retinyl oxime, dried under N_2, and resuspended in distilled water. Regeneration was achieved by the addition of the retinal analogue (concentrated solution in ethanol; final ethanol concentration in pigment, 1%) to the bacterioopsin in sodium phosphate buffer (50 mM, pH 7.2). Approximately equal molar amounts of retinal to bacterioopsin were used. After 24 h in the dark at 4 °C, the pigment was centrifuged (10000*g*, 10 min) and the pellet washed with 2% bovine serum albumin to remove excess retinal. Spectra were recorded with the bacterioopsin in the reference cell.

(*b*) *Identification of the Pigment Chromophore.* The bound chromophore was extracted from the pigment by denaturing the pigment with methylene chloride (Crouch et al., 1975; Pilkiewicz et al., 1977). Methylene chloride (3 mL) was added to the pigment (<1 mg) which had been suspended in 2 mL of water, vortexed for 1 min, and then centrifuged (10000*g* for 15 min, 4 °C). The methylene chloride layer was removed, dried over sodium sulfate, and evaporated, and the residual oil was analyzed by HPLC on a μPorasil column.

(*c*) *Stability.* The reaction of the pigment with hydroxylamine was measured by the addition of 20 μL of NH_2OH (1 M, pH 7.0) to pigment (<1 mg) and following the absorption spectrum over several hours in the dark. An assessment of the binding site of the synthetic chromophore was determined by measurement of the displacement of the analogue chromophore by addition of 5 μL of *all-trans*-retinal (0.2 mg/mL ethanol) to the synthetic pigment (1 mg in 2 mL of water) and measuring absorption over several hours. Particular note was taken of any increase in absorption at 560 nm and decrease in absorption at the γ_{max} of the analogue pigment.

(*d*) *Effect of Actinic Light on the Pigments.* Light-induced absorption changes in the submillisecond to millisecond time scale were measured with a single-beam kinetic spectrophotometer as previously described (Govindjee et al., 1980). Absorption changes were initiated with either a xenon flash (6-μs half-pulse-width) or a photoflash (200–300-μs half-pulse-width). The xenon flash was used routinely, but if no or very small signals were detected, the more intense photoflash was used. Appropriate glass cutoff filters were used to select exciting wavelengths that overlapped the major absorption band for each of the different analogue pigments. Typically

Table I: NMR Data of Retinal Analogues[a]

compound	chemical shift[b] (ppm)									
	H_{10} (d)	H_9 (dd)	H_8	7-CH_3 (s)	H_6 (d)	H_5 (dd)	H_4 (d)	3-CH_3 (s)	H_2 (d)	H_1 (d)
all-trans-**5**									5.81	10.09
all-trans-**6**					6.09	6.83	6.18	2.24	5.82	10.03
2-*cis*-**6**					6.13	6.69	6.70	2.09	5.70	10.11
all-trans-**7**			6.08[d]	2.06	6.17	6.98	6.27	2.21	5.87	10.04
2-*cis*-**7**			6.11[d]	2.04	6.23	6.81	7.00	2.07	5.66	10.09
all-trans-**8**	5.92	6.43	6.15[e]	2.01	6.13	7.06	6.25	2.30	5.82	10.01
2-*cis*-**8**	5.85	6.33	6.08[e]	2.01	6.12	6.86	7.14	2.05	5.67	10.08
6-*cis*-**8**	5.94	6.41	6.54[e]	1.98	6.05	7.17	6.28	2.32	5.81	10.00
all-trans-**9**								2.18	5.74	9.82
all-trans-**10**					1.89	6.24[c]	6.10	2.20	5.75	10.02
all-trans-**11**				1.86	6.17	6.89	6.02	2.25	5.78	10.01
2-*cis*-**11**				1.86	5.96	6.83	6.59	2.07	5.72	10.07
all-trans-**12**	1.90	5.61	6.12[e]	1.94	6.10	7.04	6.32	2.29	5.80	10.05
2-*cis*-**12**	1.91	5.62	6.09[d]	1.96	6.15	7.00	7.13	2.11	5.76	10.11

[a] Recorded on a Bruker WH-400 Fourier transform NMR spectrometer at 400 MHz and a Varian EM-390 at 90 MHz on $CDCl_3$ solutions containing $CHCl_3$ (=7.24 ppm) as internal standard. Chemical shifts are reported to a precision of ±0.01 ppm. [b] Additional chemical shifts: for **5–8**, 2′,2′-CH_3 = 0.98–1.01 (s), 3′,4′,5′-CH_2 = 0.85–0.89 (m), and 6′-CH_3 = 0.86–0.88 (m); for **9**, *trans*-3-CH_3 = 1.97 (s). [c] Multiplet. [d] Singlet. [e] Doublet.

Table II: Bacterioopsin Pigment Formation with Polyene Derivatives

compound	analogue isomer	analogue λ_{max} (mm)[a]	pigment λ_{max} (nm) da[b]	pigment λ_{max} (nm) la[b]
1	all-trans	370	560	568
5	all-trans	225		
6	all-trans	320	416	420
	2-cis	318	414	420
7	all-trans	334	458	463
	2-cis	332	455	463
8	all-trans	382	518	524
	2-cis	380	517	524
	6-cis	375	485	524[c]
9	all-trans	277		
10	all-trans	268		
11	all-trans	312	422	425
	2-cis	310	420	425
12	all-trans	347	462	466
	2-cis	346	460	466

[a] Solvent for retinals was hexane. [b] da, dark adapted; la, light adapted. [c] Isomerization to the all-trans isomer on exposure to light.

20–30 flashes were averaged in order to get good signal to noise ratios.

Flash-induced proton release and uptake was measured as described earlier (Govindjee et al., 1980) by observing the absorption changes of the pH-sensitive dye *p*-nitrophenol. In most cases the monitoring wavelength was 395 nm, but to avoid a flash artifact problem with the pigment formed from compound **7**, the measurements were made at 315 nm (Figure 2a). In all cases the pH was 6.8 ± 0.05, and the salt concentration was 150 mM KCl. Since the extraction coefficients of the artificial pigments are unknown, no precise quantitative comparison of proton release and uptake efficiencies can be made.

Results

Two series of compounds were synthesized: a group containing the trimethylated cyclohexyl ring but with the length of the polyene chain varied (**5–8**) and a selection of polyene compounds that lack any portion of the cyclohexyl ring (**9–12**). Both series contain methyl groups on the polyene chain that are in the same position with respect to the aldehyde function as the methyls on the retinal side chain. The 2-cis isomers correspond to the 13-cis isomer of retinal. For the purpose of discussion, the carbons considered as being in the polyene chain are only those involved in the backbone of the chain and do not include methyl substituents or cyclic carbons except those directly extending the length of the chain. Spectral data for the retinals are summarized in Tables I and II.

Compounds **5** *and* **6**. Compound **5**, containing one ethylenic bond in the side chain, shows no association with bacterioopsin. However, the all-trans and 2-cis isomers of compound **6** with three ethylenic bonds in the side chain do form pigment complexes with bacterioopsin. The absorption maxima are at approximately 410–416 nm. The pigments demonstrate a shift on light adaptation and have some stability to both hydroxylamine and *all-trans*-retinal. However, when tested for both a photocycle and light-activated proton release, neither function was observed (Table III).

Compound **7**. Compound **7** contains four ethylenic bonds in the side chain and is one carbon shorter than retinal itself. Both the all-trans and 2-cis isomers of **7** form stable pigments with bacterioopsin. The absorption maxima are at approxi-

Table III: Absorption and Photocycle Data of Bacterioopsin Analogue Pigments[a]

retinal	cyclohexyl ring	no. of ethylenic bonds in polyene chain	pigment da λ_{max} (nm)	pigment da $\Delta\nu$ (cm^{-1})	pigment la λ_{max} (nm)	"M" photointermediate λ_{max} (nm)	"M" photointermediate $\Delta\nu$ (cm^{-1})	H^+ release and uptake
1	+	4	560	6780	568	412	4740	++
5	+	1				NA		NA
6	+	3	416		420			–
7	+	4	458	6180	463	355-360	4720	+
8	+	5	518	6230	524	390-395	4500	++
9	–	1				NA		NA
10	–	2				NA		NA
11	–	3	422		425			–
12	–	4	462	6370	466	365	4500	+

[a] NA, not applicable; da, dark adapted; la, light adapted; (+) moderate pumping observed with photoflash; (–) no pumping with xenon flash; (++) strong pumping observed with both photoflash and xenon flash; $\Delta\nu$, bandwidth.

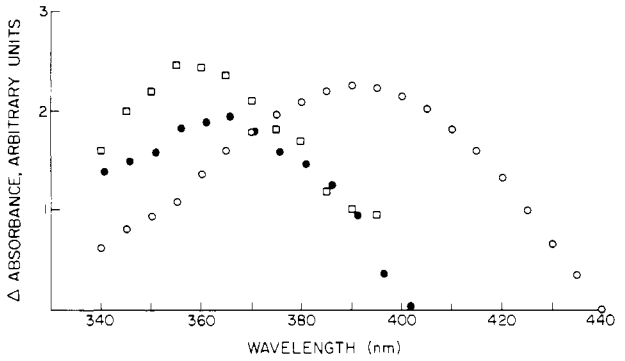

FIGURE 1: Flash-induced difference spectrum of analogue pigments in 150 mM KCl; pH 6.8 ± 0.05; temperature approximately 20 °C; actinic flash >430 nm (xenon flash plus Corning CS3-72 filter). (□) 7-based pigment; (○) **8**-based pigment; (●) **12**-based pigment.

mately 458 nm (Table II). Both pigments have reasonable stability to hydroxylamine ($t_{1/2}$ of degradation = 70 min). However, the yield of pigment regeneration is rather low and the formation time slow ($t_{1/2}$ = 25 min). Excess bacterioopsin is found to be present after regeneration because, upon addition of *all-trans*-retinal, bacteriorhodopsin (λ_{max} at 560 nm) is formed immediately without any loss of analogue pigment absorption. Eventually the excess *all-trans*-retinal causes a decrease in the absorption of the pigment at 458 nm and a further increase in bacteriorhodopsin absorption at 560 nm. Thus, the analogue chromophore seems to bind at the same site as *all-trans*-retinal but with lower affinity. The analogue pigment shows light/dark adaptation with a 5-nm red shift in absorption maximum for the light-adapted form.

The all-trans analogue pigment undergoes a photocycle. The flash-induced difference spectrum has a maximum around 355–360 nm [Figure 1 (□); Table III]. The M-type intermediate decays with a half-time of approximately 11 ms. The pigment shows light-induced proton release with a rather low yield (Figure 2a).

Compound **8**. The 2-cis and all-trans isomers of compound **8** with five ethylene bonds in the side chain form stable pigments with bacterioopsin within approximately 2 min. The absorption maxima of both pigment analogues are approximately 518 nm. The yield of pigment regeneration is very high; little or no free bacterioopsin was detected in the presence of a small excess of the chromophore because no absorption increase at 560 nm was observed upon addition of *all-trans*-retinal. The binding affinity of the analogue compound is very high as almost no displacement of the analogue compound was observed upon addition of excess *all-trans*-retinal. The pigment is stable to hydroxylamine for several hours. Upon light adaptation, the absorption maximum shifts to approximately 524 nm. The analogue pigment undergoes photocycling and shows proton release with high yield (Figure 2b). The flash-induced difference spectrum has a maximum at approximately 395 nm [Figure 1 (○); Table III]. The M-type photointermediate decays with a half-time of approximately 11 ms at 20 °C, pH 6.8.

The 6-cis isomer of **8** (corresponding to the 9-cis isomer of retinal) likewise forms a pigment with bacterioopsin with a λ_{max} of 485 nm. On exposure to light, the γ_{max} shifts to 524 nm. On dark adaptation of the 524-nm pigment, the absorption shifts only 8 nm to 516 nm. Extraction of the chromophore from the pigment that had never been exposed to light showed it to still be the 6-cis isomer. Chromophore extracted from the light-adapted pigment was in the all-trans isomeric form. After dark adaptation of the pigment previously exposed to light, a mixture of the 2-cis and all-trans

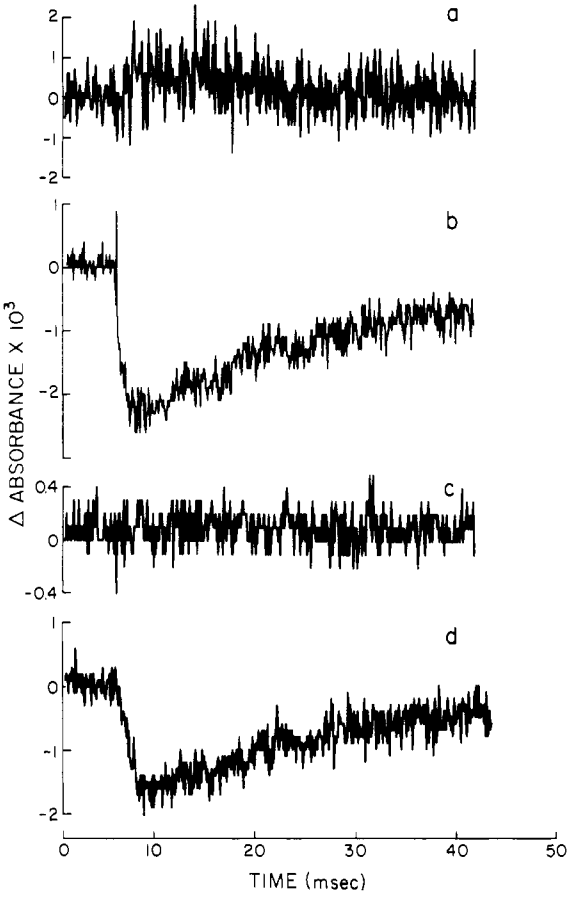

FIGURE 2: Flash-induced absorbance change of the pH-sensitive dye *p*-nitrophenol in the presence of the following: (a) analogue pigment **7**, measuring λ = 315 nm and actinic flash λ > 400 nm (photoflash plus Corning CS3-73 filter; note that at this monitoring wavelength for *p*-nitrophenol proton release and uptake have an opposite sign than at the more usual 395 nm); (b) analogue pigment **8**, measuring λ = 395 nm and actinic flash λ > 460 nm (xenon flash plus Corning CS3-71 filter); (c) analogue pigment **11**, measuring λ = 395 nm and actinic flash λ > 400 nm (photoflash plus CS3-73 filter); (d) analogue pigment **12**, measuring λ = 395 nm and actinic flash λ > 430 nm (xenon flash plus Corning CS3-72 filter). All samples, pH 6.8 ± 0.05 and temperature approximately 20 °C.

isomers of **8** was obtained. These results are similar to those obtained for the acyclic retinals (Crouch et al., 1984).

Compounds **9** *and* **10**. Polyene compounds **9** and **10** with no part of the cyclohexyl ring present, containing one and two ethylenic bonds, respectively, did not form pigments with bacterioopsin.

Compound **11**. The 2-cis and all-trans isomers of polyene compound **11** containing three ethylenic bonds do form pigments with bacterioopsin with absorption maxima at approximately 420 nm ($t_{1/2}$ = 20 min). The yield of pigment regeneration was very low. The pigments were moderately stable to hydroxylamine and to displacement by *all-trans*-retinal (45–60 min). Pigment-formed **11** did not show any flash-induced absorption changes (data not shown) and does not appear to be able to support light-activated proton release.

Compound **12**. The 2-cis and all-trans isomers of polyene compound **12** with four ethylenic bonds formed a stable pigment with absorption maximum at approximately 460 nm. The pigments formed quickly ($t_{1/2}$ = 10 min) and were reasonably stable to the addition of hydroxylamine and *all-trans*-retinal ($t_{1/2}$ of displacement = 90 min). The all-trans pigment showed photocycling and light-induced proton release [Figure 1 (●)]. The flash-induced difference spectrum has a maximum at approximately 365 nm (Figure 2d; Table III).

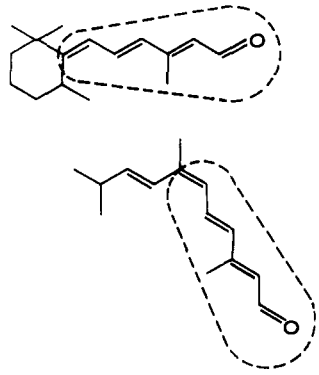

FIGURE 3: Structure of 6-*cis*-**6** and 6-*cis*-**2** demonstrating the trans configuration of three ethylenic bonds with respect to one another.

Discussion

Previous studies have shown that numerous retinal analogues form pigments with bacterioopsin and, of those tested, the majority of the pigment analogues undergo photocycling and show light-activated proton pumping and/or release. A role for the cyclohexyl ring in this function has been eliminated as pigments regenerated with acyclic retinal analogues **2**–**4** show photochemistry and efficient light-activated proton release (Crouch et al., 1984).

The results reported here show that indeed pigments can be formed with aldehyde polyenes containing at least three (trans) ethylene bonds in the polyene chain. The formation of pigment from 7-*cis*-retinal (Kohl & Sperling, 1979) is probably due to the presence of the three trans bonds, for which **11** is a model. Likewise, the association of **6** and the 6-cis isomers of acyclics (Crouch et al., 1984) with bacterioopsin can be explained by the presence of three ethylene bonds that are trans to one another (Figure 3). These pigments do not appear to photocycle or show light-activated proton release. However, if the polyene chain contains four ethylenic bonds (**7** and **12**), a pigment is formed with photocycles, has an M intermediate, and releases protons after a flash, although not with the same efficiency as in bacteriorhodopsin. Moreover, on all of the pigments examined here, both the half-times of proton uptake and M decay (data not shown) are substantially longer than other native bacteriorhodopsin or regenerated bacteriorhodopsin. The addition of one more carbon (retinal, **1**) completely restores the light-activated proton release function. Derivatives with the full polyene chain including four ethylenic bonds and some skeleton of the ring [e.g., the acyclics (Crouch et al., 1984)] likewise are fully functional in proton release. Interestingly, one extra carbon (**8**) in the retinal polyene chain does not diminish this activity.

The M-type intermediates of pigments **7**, **8**, and **12** have properties similar to that of the retinal- (**1**) based pigments. The bandwidths of all four pigments are similar (4500–4740 cm^{-1}) in contrast to the narrow bandwidth naphthyl pigments (Crouch et al., 1985). The wavelength shifts (in energy units) seen when the pigment is converted to its M intermediate are also surprisingly similar, although the retinal- (**1**) based pigment does have a slightly larger shift than the artificial pigments.

The cyclohexyl ring has no role important in the physiological functions of bacteriorhodopsin. The relationship of the methyl groups to the Schiff base is the same in these analogues as for retinal itself. Therefore, the only structural component being varied with this series of compounds (**5**–**12**) is the length of the polyene chain. Indeed, these results demonstrate that the length of the polyene chain is crucial to the photocycling and photon photorelease functions of the bacteriorhodopsin and that although synthetic pigments may be generated with a number of analogues, the protein has little tolerance for changes in this critical structural feature for the formation of a physiologically active pigment.

Added in Proof

Compounds **11** and **12** have been reported to form pigments with bacterioopsin (Muradin-Szweykowska et al., 1984). However, our results here and the results reported in Crouch et al. (1984) do not support the conclusions of these workers that five conjugated double bonds or the cyclohexyl ring are required for proton pumping. In contrast to Muradin-Szweykowska et al., we did obtain proton pumping from the pigment formed with **12**, an acyclic compound containing four double bonds.

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Registry No. **1** (all-trans), 116-31-4; **5** (all-trans), 100513-69-7; **6** (all-trans), 100513-70-0; **6** (2-cis), 100513-71-1; **7** (all-trans), 100513-72-2; **7** (2-cis), 100678-57-7; **8** (all-trans), 100513-73-3; **8** (2-cis), 100678-58-8; **8** (6-cis), 100678-59-9; **9**, 107-86-8; **10** (all-trans), 69793-56-2; **11** (all-trans), 85441-31-2; **11** (2-cis), 49831-80-3; **12** (all-trans), 89435-52-9; **12** (2-cis), 100678-60-2; C_5 phosphonate, 41891-54-7; 2,2,6-trimethylcyclohexanone, 2408-37-9; vinylmagnesium bromide, 1826-67-1; 1-vinyl-2,2,6-trimethylcyclohexanol, 6221-20-1; triethyl phosphonoacetate, 867-13-0; 3-(2′,2′,6′-trimethylcyclohexylidene)propan-2-one, 100513-74-4; ethyl 4,4-(2′,2′,6′-trimethylcyclohexylidene)-3-methyl-2,4-butenoate, 100513-75-5; ethyl 3,7-dimethyl-2,4,6,8-decatetraenoate, 100570-85-2; hydrogen ion, 12408-02-5.

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Evidence for Processing of Maize Catalase 2 and Purification of Its Messenger RNA Aided by Translation of Antibody-Bound Polysomes†

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ABSTRACT: Two-dimensional gel analysis of the in vitro and in vivo labeled catalase 2 (CAT-2) isozyme protein of *Zea mays* L. and western gel analysis of native CAT-2 and in vitro labeled CAT-2 indicated that the protein is processed from a precursor to a lower molecular weight form in the scutellum. The CAT-2 from each source appeared on two-dimensional gels as one major species and two to three subspecies of the same molecular weight. We have also purified the mRNA encoding CAT-2 from scutella of line R6-67 using the procedure of polysome immunoadsorption. As a midcourse check on the progress of purification, we translated a small portion of the purified *Cat2* mRNA-containing polysomes while they were still complexed with CAT-2 antibodies and bound to protein A–Sepharose. This revealed the presence of highly purified *Cat2* polysomes. The final mRNA could not be translated in the wheat germ system but was highly active in the reticulocyte lysate system. The translation product had a molecular weight of 56 000, compared to that of 54 000 for purified CAT-2 protein. We have also enriched for *Cat2* mRNA by size selection on methylmercury–agarose gels. The *Cat2* resided with and slightly above the 18S ribosomal contaminant band of the total poly(A+) mRNA. It is therefore about 1805 bases long, which is 224 bases longer than the calculated coding length of 1581 bases.

The enzyme catalase (CAT; EC 1.11.1.6; H_2O_2:H_2O_2 oxidoreductase) of maize (*Zea mays* L.) exists as a tetramer of 54-kDa[1] subunits held together without disulfide bonds. Three electrophoretically distinct isozymes of maize CAT have been identified and shown to be encoded in three unlinked genes (Scandalios et al., 1980a; Scandalios, 1983). The three structural genes (*Cat1*, *Cat2*, and *Cat3*) have been mapped to different locations within the maize genome (Roupakias et al., 1980). In this study, we have focused on the CAT-2 isozyme as part of an effort to understand the developmental regulation of its synthesis.

In the scutellum of certain genetic lines, during early seedling growth, the product of a trans-acting temporal regulatory gene (*Car1*) acts to maintain an elevated level of CAT-2, which would otherwise decline after 4 days from imbibition (Scandalios et al., 1980b). CAT-2 is also inducible by chemical and environmental stimuli (including light in leaves) (Scandalios, 1983) and displays cell-type specificity (Tsaftaris et al., 1983), tissue specificity (Scandalios, 1979), and organellar targeting (Scandalios, 1974).

In order to further study these phenomena, we have enriched for *Cat2* mRNA sequences by polysome immunoadsorption and by size fractionation of total poly(A+) mRNA. In the course of these studies we have determined the approximate size of the *Cat2* mRNA and its concentration as a percent of total translatable mRNA. Evidence for processing in vivo is presented, as well as observations of maize mRNA translation and polysome immunoadsorption. Of particular usefulness was the finding that the *Cat2*-containing polysomes could be translated in situ while still bound to protein A–Sepharose beads via CAT-2 antibodies. This is a useful technique to employ as a midcourse check for those wishing to purify mRNAs by polysome immunoadsorption.

Materials and Methods

Materials. RNase-free sucrose and reticulocyte lysates were purchased from Bethesda Research Laboratories, protein A–Sepharose was from Pharmacia, low melting point agarose was from Marine Colloids, oligo(dT)–cellulose was from Collaborative Research, and nitrocellulose filters were from Schleicher and Schuell. All other chemicals were reagent-grade, purchased from Sigma Chemical Co.

Polysome Immunoadsorption. Seeds of the high CAT-2 activity line R6-67 (Scandalios et al., 1980b) were surface-

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[1] Abbreviations: Da, dalton; 2-D, two-dimensional; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVP-40, polyvinylpyrrolidone-40; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; CAT-1, CAT-2, and CAT-3, isozymic forms of maize catalase; *Cat1*, *Cat2*, and *Cat3*, structural genes encoding for the three catalase isozymes, respectively; RNase, ribonuclease; Tris, tris(hydroxymethyl)aminomethane; PPO, diphenyloxazole; IgG, immunoglobulin G; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid.

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