is more favorable than electron transfer in the opposite direction. This conclusion rests on a comparison of the experimentally observed spectra with the calculated spectra that are obtained under various assumptions concerning the CT energies. It also is in harmony with the sensitivity of the long-wavelength absorption band to external electric fields.

The formalism that we have developed here can be extended straightforwardly to explore the mechanism of the photochemical electron-transfer reaction. Preliminary results, reported in ref 51 and 56, give a coupling matrix element of 5.9 cm^{-1} for the formation of $P^+BChl_{LA}^-$ from the excited reaction center (P*), and 15 cm^{-1} for the subsequent hopping of an electron to BPh_L. An alternative intermediate CT state, $BChl_{LA}^+BPh_L^-$, can be generated from P* with a matrix element of 2.5 cm^{-1} and then relax to $P^+BPh_L^-$ with a matrix element of 11 cm^{-1}.

Acknowledgment. This work was supported by National Science Foundation Grants PCM-8303385, PCM-8312371, and PCM-8316161. We also acknowledge with thanks the help of the ISIS program from Digital Equipment Corp. in obtaining the MicroVax II computer on which the calculations were done. We thank J. Deisenhofer and H. Michel for providing the crystallographic coordinates and J. Breton, D. Holten C. Kirmaier, M. Gouterman, D. Middendorf, R. Pearlstein, and A. Scherz for helpful discussion.

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A Rhodopsin Pigment Containing a Spin-Labeled Retinal

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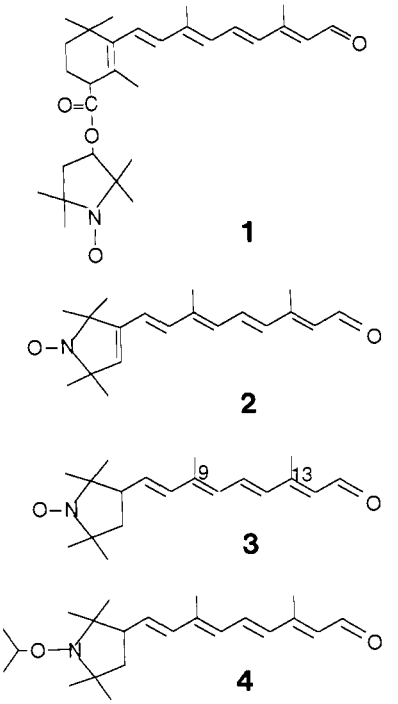
Abstract: A retinal derivative containing a nitroxide group has been synthesized and a photosensitive pigment formed between the 9-cis isomer and bovine opsin. The pigment is stable to hydroxylamine and 11-*cis*-retinal. The electron spin resonance spectrum of the pigment in suspension shows that the nitroxide is strongly immobilized ($\tau > 10^{-7}$ s), is inaccessible to small hydrophilic reagents, and is not highly oriented within the membrane. The electron spin resonance spectrum of detergent-solubilized pigment is highly sensitive to the nature of the detergent, the digitonin and CHAPS solubilized pigment showing spectra most closely resembling the spectrum of pigment in suspension. These data are consistent with the ring portion of the retinal chromophore being deep within the membrane in a hydrophobic environment.

The use of synthetic analogues of retinal to probe the binding site and photochemistry of both the visual pigment rhodopsin and bacteriorhodopsin from the purple membrane of *Halobacterium halobium* has been well established. This approach has been used to ascertain the minimal structural requirements for pigment formation[1,2] and has contributed significantly to understanding of the primary photochemical event in vision.[3] Analogues incorporating the nitroxide "reporter" group are especially well suited to study the protein environment of the chromophore and the motional behavior of these membrane proteins through the use of electron spin resonance (ESR) spectroscopy.[4] We have used this approach to study the binding site of the purple membrane with a spin-labeled derivative of retinal (**1**) in which the nitroxide-containing ring was joined to the retinal ring by an ester linkage[5] and with a second derivative **2** incorporating the nitroxide into the retinal itself, which more closely resembled the parent molecule.[6] The ester **1** was limited in application by protein-mediated hydrolysis which liberated the nitroxide from the membrane. The second nitroxide derivative **2** formed a stable bacteriorhodopsin pigment but failed to combine with bovine opsin to form a pigment (unpublished results).

We report here the synthesis of a new spin-labeled retinal (SLR) **3** that differs from the previous analogue **2** only in that the nitroxide-containing ring lacks a carbon–carbon double bond. This



modification in the structure of the SLR now allows the 9-cis isomer to form a stable pigment when combined with bovine opsin, representing the first report of an opsin pigment containing a spin-labeled retinal. This product fulfills the standard criteria for stable photosensitive rhodospin analogue pigments, and the ESR spectrum is consistent with the photochemical behavior. The retinal binding site is shown to be hydrophobic in nature and excludes water from the ring end of the chromophore. The temperature dependence of the ESR spectrum and the effects of detergent solubilization on the ESR data are described.

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Table I. NMR Data of Isopropyl Derivatives of the Spin-Labeled Retinal 4 Isomers

isomer	chemical shift, ppm							
	13-CH_3 (s)	H_7 (m)	H_8 (d)	H_{10} (d)	H_{11} (dd)	H_{12} (d)	H_{14} (d)	H_{15} (d)
all-trans	2.30	5.70	6.13 (15.0)	6.13 (11.3)	7.07 (11.38 15.08)	6.34 (15.0)	5.95 (8.5)	10.08 (8.5)
13-*cis*	2.12	5.71	6.15 (15.4)	6.17 (11.3)	6.97 (11.3, 15.0)	7.23 (15.0)	5.82 (8.2)	10.17 (8.2)
9-cis	2.32	5.73	6.63 (15.1)	6.03 (11.5)	7.19 (11.5, 15.0)	6.28 (15.0)	5.95 (8.2)	10.08 (8.2)
9,13-di-cis	2.13	5.73	6.62 (15.3)	6.06 (11.5)	7.05 (11.5, 15.3)	7.10 (15.3)	5.83 (8.0)	10.17 (8.0)

[a] Spectra recorded on a Bruker WH-400 Fourier transform NMR spectrometer at 400 MH on solutions in $CDCl_3$ containing $CHCl_3$ (7.24 ppm) as internal standard. Coupling constants (Hz) in parentheses. Chemical shifts are reported to a precision of ±0.01 ppm and coupling constants to ±0.2 Hz. Additional chemical shifts: 9-CH_3, 1.95 (s); ring methyls, 1.16–1.18 (s); isopropyl ON group, CH_3 (2) 0.90, 0.91 (d, J = 6.7); methine H, 3.50 (m).

Experimental Section

All experiments on retinals and regenerated pigments were carried out under dim red light. The retinal syntheses were conducted under nitrogen and the retinals stored at −70 °C under argon. Absorption spectra were recorded on a Varian 2200 double-beam spectrophotometer equipped with a cell stirrer and temperature controller using black walled-quartz cuvettes (1.5 mL). All pigment spectra were obtained in sodium phosphate buffer (67 mM, pH 7.4) with opsin as the reference. Retinal spectra were obtained in ethanol (EtOH) with solvent as the reference. Nuclear magnetic resonance (NMR) spectra were obtained on Bruker WH-400 NMR spectrometer (obtained at the National Science Foundation facility at the University of South Carolina, Grant CHE 7818723). The NMR data for all compounds are presented in Table I. Mass spectra were obtained on a Finnigan 3200 with a direct probe using electron impact (70 eV).

Synthesis of Spin-Labeled Retinal. Solvents were dried immediately prior to use. Tetrahydrofuran (THF) was dried and distilled from a purple solution of benzophenone and sodium; methylene chloride was refluxed and distilled from phosphorus pentoxide. Triethylamine was dried and distilled from potassium hydroxide. Dimethyl sulfoxide (Aldrich, anhydrous, 99+%) and oxalyl chloride (Aldrich, 99+%) were used without further purification. Ethyl 4-(dimethoxyphosphinyl)-3-methyl-2-butenoate was prepared according to literature procedure[8] and distilled under reduced pressure. Analytical thin-layer chromatography was done on (2.5 × 10 cm) plates precoated with 0.25-mm-thick silica gel with fluorescent indicator made by Analtech; similar, but larger (10 × 20 cm) glass plates were used for preparative purposes. Visualization was accomplished with a 7% solution of phosphomolybdic acid in 95% ethanol or with short-wavelength UV light. Silica gel, 80–140 mesh, supplied by J. T. Baker Chemical Co., was used for column chromatography.

2,2,5,5-Tetramethylpyrrolidinyl-1-oxy-3-methanol (6). Ethyl chloroformate (5.78 mmol, neat) was added to a solution of 2,2,5,5-tetramethylpyrrolidinyl-1-oxy-3-carboxylic acid (Eastman, 5.37 mmol) and triethylamine (5.75 mmol) in THF (25 mL) without external cooling. After the mixture was stirred at room temperature for 1 h, triethylamine hydrochloride was removed by filtering the reaction mixture through a short silica gel column and eluted with more THF (10 mL). The solution containing the mixed anhydride was then added dropwise to a stirred suspension of lithium aluminum hydride (LAH, 10 mmol) in THF (5 mL) under argon at 0 °C. After 30 min at 0 °C, water (0.4 mL) was slowly added via syringe and the mixture stirred at room temperature overnight. The precipitate was removed by centrifugation (8000g, 5 min) and extracted four times with ether. The combined organic phases were dried over sodium sulfate, and solvent was removed to give the alcohol as a bright yellow solid;[7] 87% yield; m/e 172 (M^+).

2,2,5,5-Tetramethylpyrrolidinyl-1-oxy-3-carboxaldehyde (7). A solution of dimethyl sulfoxide/oxalyl chloride complex was prepared by adding dimethyl sulfoxide (14.3 mmol) in methylene chloride (6 mL) dropwise to a solution of oxalyl chloride (7.1 mmol) in methylene chloride (6 mL), cooled in dry ice–carbon tetrachloride bath. The rate of addition was adjusted such that the internal temperature would not exceed 20 °C. The resulting reaction mixture was stirred with cooling for an additional 40 min to ensure complete complex formation. Alcohol **6** (3.5 mmol) in methylene chloride (3 mL) was added dropwise to the complex solution. The reaction mixture became homogeneous in 5 min. After 20 min, triethylamine (14 mmol) in methylene chloride (1 mL) was added dropwise via syringe; the internal temperature was maintained around 20 °C during the addition. The cooling bath was removed after 10 min, the reaction mixture was allowed to warm to room temperature, water (20 mL) was added, the aqueous phase was extracted with methylene chloride (2 × 10 mL) and dried over sodium sulfate, and solvent was removed under vacuum. The aldehyde **7** was purified by column chromatography (silica gel, 60 g; 30% ether/hexane): 92% yield; m/e 170 (M^+).

3-Methyl-5-(2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)penta-2,4-dienoic Acid, Ethyl Ester. Condensation of aldehyde **7** (3 mmol) with the ethyl 4-(dimethoxyphosphinyl)-3-methyl-2-butenoate[8] (3 mmol) was accomplished in the presence of sodium hydride (2 × 3 mmol) in THF (20 mL) by stirring at room temperature for 20 h under argon. The excess hydride was destroyed by cautious addition of water (10 mL), and the mixture was taken up into a 1/1 water/ether mixture. The aqueous phase was further extracted with ether. The combined organic phase was washed once with brine and dried over sodium sulfate and solvent removed under vacuum. Purification by column chromatography (silica, 80 g; 40% ether/hexane) gave the ester as a mixture of two isomers: 83% yield; m/e 280 (M^+); λ_{max} 260 nm.

3-Methyl-5-(2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)penta-2,4-dienal (5). A solution of ester (2.18 mmol in 5 mL of THF) was added into a suspension of LAH (2.5 mmol) in THF (10 mL) cooled in a dry ice–acetone bath under argon. After the addition, the cooling bath was replaced with ice–water. The reaction was followed by TLC (40% ether/hexanes) of aliquot workup and with an aliquot reaction mixture in ethanol by observing the disappearance of λ_{max} at 260 nm and the formation of a new λ_{max} at 245 nm. Excess hydride was destroyed with water (0.1 mL), and the precepitate was removed by centrifugation and washed throughly with ether (4 × 15 mL). The combined solution was dried over sodium sulfate and solvent removed under vacuum. The alcohol was purified by column chromatography (silica, 30 g; ether) and used without further characterization. The alcohol was then oxidized with activated MnO_2 (5 mmol) and THF (10 mL) at room temperature overnight. Solid was removed by centrifugation and washed with ether (3 × 15 mL). The aldehydes were purified via flash column chromatography on silica gel (50 g) with 30% ether/hexanes followed by preparative thin-layer chromatography (40% ether/hexanes) to give the less polar component as 2-*cis*-**5** in two steps: 28% yield m/e 236 (M^+); λ_{max} 284 nm.

3,7-Dimethyl-9-(2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)nona-2,4,6,8-tetraenal (3). The final condensation of the phosphonate with each of the aldehydes **5** and the succeeding reduction and oxidation to yield the product in 52% yield were as described for the sequence. The isomers [m/e 302 (M^+)] were separated by preparative thin-layer chromatography (30% ether/hexanes) to yield 9-*cis*-**3** (λ_{max} 355 nm), 9,13-di-*cis*-**3** (λ_{max} 356 nm), 13-*cis*-**3** (λ_{max} 360 nm), and *all-trans*-**3** (λ_{max} 362 nm). By assuming the extinction coefficient to be the same as that of *all-trans*-retinal and calculating the concentration of nitroxide groups by calibrating with a standard compound, 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy, one unpaired electron was found per molecule. The stereochemistries of the aldehydes were determined by the NMR spectra (Table I) of the corresponding isopropoxyamines prepared by the following procedure: diisopropylaluminum hydride (Aldrich, 1.0 M in THF, 0.1 mmol) was added to the aldehyde (0.1 mmol) in THF (1 mL) at 0 °C, and the resultant mixture was stirred at room temperature until the solution turned from yellow to colorless (approximately 1 h). Water (50 μL) was added and the mixture stirred at room temperature for 2 h. Gel was removed by centrifugation and the supernatant washed with ether (3 × 10 mL). The combined organic phases were dried over sodium sulfate, and solvent was removed under reduced pressure. The product was examined for purity by thin-layer chromatography analysis and for the absence of spin by ESR. The alcohol was then oxidized with activated MnO_2 to give the corresponding aldehyde [e.g., **4** in 67% yield, λ_{max} 360 nm, m/e 343 (M+)]. Simultaneous thin-layer chromatography analysis (40% ether/hexanes) of the corresponding crude aldehydes showed no sign of double-bond isomerization.

Pigment Studies. The method of preparation of the bovine rod outer segments (ROS) was essentially that of Papermaster and Dreyer.[9] Yields are quantitated as the difference in optical density at the λ_{max} (498

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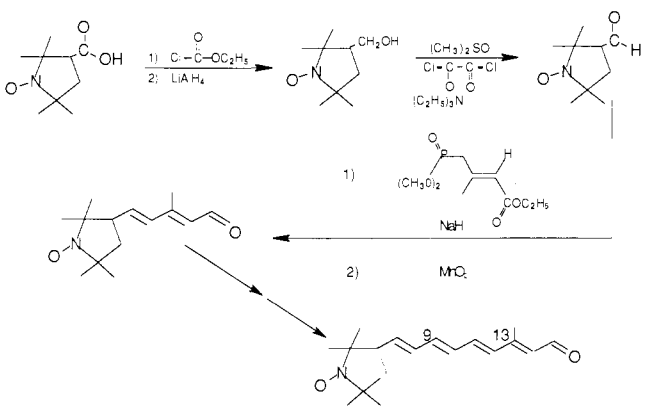

Figure 1. Reaction scheme for formation of spin-labeled retinal.

nm) before and after complete photobleaching and converted to milligrams of rhodopsin with the extinction coefficient 40000.[10]

Rhodopsin was bleached by suspending ROS (1 mg) in 3 mL of phosphate buffer (67 mM, pH 7.4) with hydroxylamine (NH_2OH, 20 mM) and exposing it to intense white light at 0 °C through a UV-absorbing filter for 90 s. The bleached ROS were then sedimented by centrifugation (37000*g*, 3.5 min), resuspended, and washed by spinning as above, three times with 2% bovine serum albumin in buffer (67 mM, pH 7.4) and three times in buffer alone. For pigment formation, 30 μL of a solution of the 9-*cis*-SLR in EtOH to yield a 5-fold excess of the SLR was added with stirring at 4 °C to the suspension of bleached ROS in 3 mL of buffer. After a 12–18-h incubation, the ROS were sedimented and washed as for bleaching above, except that the first wash in BSA also contained 20 mM NH_2OH and was allowed to incubate at 0 °C for 15 min before subsequent centrifugation in order to assist in the removal of excess SLR. Regeneration yields of SLR pigment generally approached those with 9-*cis*-retinal, assuming similar extinction coefficients. Excess 11-*cis*-retinal in EtOH was added to the SLR pigment and the visible absorbance spectrum monitored for 1 h. There was no appearance of an absorbance peak at 498 nm (λ_{max} for rhodopsin). The addition of NH_2OH to the SLR pigment in the dark did not affect the absorbance spectrum.

For experiments with detergent, ROS membranes regenerated with 9-*cis*-RSL were resuspended in buffer containing one of the following detergents: 1% Triton X-100, 1% digitonin (recrystallized from H_2O),[11] 1% Ammonyx LO or 1% 3-[(3-chloramidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS).[12] ROS were disrupted with three to five short (1–3-s) bursts of sonication at less than 50 kW with a Heat Systems sonifier with a microtip. Insoluble material was sedimented by spinning at 39000*g* for 30 min.

EPR Measurements. Conventional (V'_1) EPR experiments were performed on a Varian E-4 spectrometer equipped with a standard E 102 cavity and a Varian variable-temperature controller. Temperature was monitored by a copper/constan thermocouple and temperature-calibrated potentiometer. The EPR was interfaced to a DEC PDP 11/23 computer by analog to digital convertor. Data were digitized, stored, and manipulated with software developed by G.E.R. Samples were measured in a standard quartz flat cell (200 μL) at room temperature. Samples at other than room temperature were measured in a special flat cell (30 μL). Irradiation in the EPR cavity was accomplished with fiber optics to isolate the sample from the heat of the light source. For orientation experiments, films were prepared by placing resuspended ROS on a quartz flat plate and drying in vacuo until no visible moisture remained. Spectra were obtained with the sample cavity exposed to room air.

Results and Discussion

Synthesis and Characterization of Spin-Labeled Retinal. The synthetic pathway used for the SLR analogue **3** is shown in Figure 1. The starting carboxylic acid was resistant to the conventional reduction/oxidation sequence of $LiAlH_4/MnO_2$ used in subsequent steps, probably due to the presence of lithium salts. The application of the ethyl chloroformate/$LiAlH_4$ method according to the general procedure of Schipper and Nichols[13] was used to obtain the alcohol followed by the Swern method[14] of oxidation to obtain the aldehyde. The new step was able to accomplish the reaction at the alkyl carbonyl without adversely affecting the nitroxide unlike other less gentle methods used to coax this type of carbonyl to reduce. The remainder of the synthetic sequence is conventional isoprenoid procedures frequently used in previous analogue work. All intermediates were identified by mass, NMR, and absorption spectroscopies. The intermediate aldehyde was obtained as two isomers, cis and trans at the C-9 position.

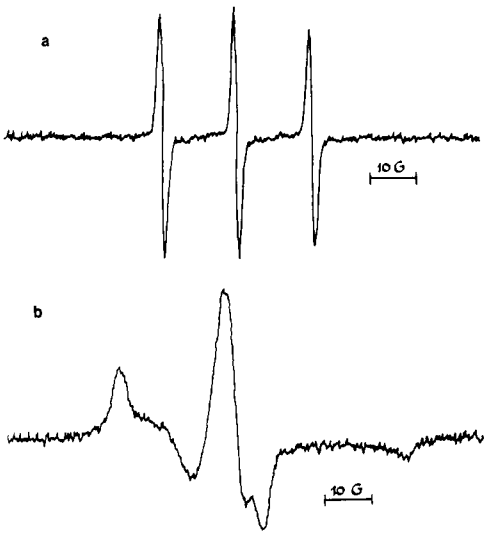

Figure 2. ESR (V'_1) spectra: (a) SLR in solution, in EtOH (less than 1%) dispersed in 67 mM phosphate (pH 7.4) at 20 °C; (b) SLR pigment, dark-adapted in 67 mM phosphate buffer. Conditions: $2A_{zz}$ = 64.1 G; a_0 = 16 G; modulation amplitude 1.25 G; microwave powder 7.5 mW; 22 °C.

Mass spectrometry of the final product revealed a parent peak at *m/e* 302 as well as several major peaks consistent with the products of breakage of the SLR at various key points, such as the ring/side chain bond. Four isomers of **3** were obtained that were separated and shown to have λ_{max} at 365–368 nm, consistent with the abbreviated conjugated double-bond system of the SLR relative to retinal (see Figure 3, curve a, for the 9-cis isomer).

The purified SLR intermediates and final products were characterized by NMR spectrometry. Because the nitroxide free radical readily undergoes electron–electron nuclear dipolar interactions with a broad array of nuclei, the NMR spectrum is severely broadened to the point where no meaningful interpretation is possible from lack of resolution of the peaks. Characterization by NMR spectrometry was made feasible by the reaction with diisopropylaluminum hydride to form the isopropyl derivative at the nitroxide nitrogen. This method was gentle with a minimum of side reactions and effectively retained the isomeric configuration of the analogue while eliminating the paramagnetic center. In this manner all proton peaks in the NMR spectra were unambiguously assigned, and the cis/trans isomeric configuration at each carbon–carbon double bond was clearly determined (Table I).

The EPR spectrum of the SLR (Figure 2a) showed a typical triplet nitroxide spectrum with slight attenuation of the upfield resonance due to the effect of the length of the molecule on its tumbling behavior. The presence of one unpaired electron per SLR molecule was calculated by double integration of the ESR spectrum.

Formation and Characterization of Spin-Labeled Isorhodopsin Pigment. When the 9-*cis*- or 9,13-di-*cis*-SLR is added to a suspension of opsin, a pigment is formed that absorbs visible light with λ_{max} 448 nm (Figure 3, curve B). The pigment fulfills the requirements of a true rhodopsin analogue pigment in that when 11-*cis*-retinal is added to the suspension, no regeneration of rhodopsin is observed as determined by lack of increase in absorbance at 498 nm and no displacement of SLR occurs as the

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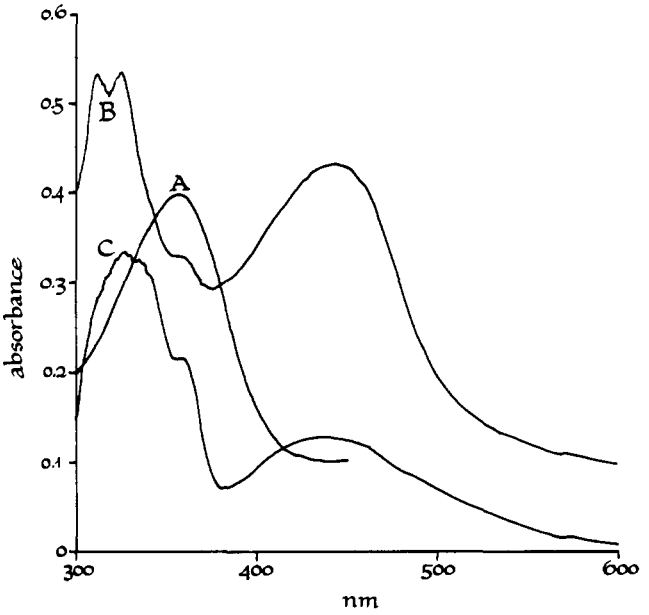

Figure 3. Absorption spectra: (a) 9-*cis*-SLR in EtOH; (b) SLR pigment with opsin used as reference; (c) SLR pigment after 90-s illumination in 20 mMol NH_2OH.

absorbance at 448 nm is not decreased. These results show that the SLR occupies the unique binding site normally filled by retinal in the native pigment and is attached to the same lysine residue as the native chromophore.[15] The SLR pigment is stable in the dark in the presence of 20 mM NH_2OH, indicating that the Schiff base linking the SLR to the protein is protected from the aqueous environment as in the native pigment. If the pigment is illuminated in the presence of NH_2OH, the visible absorbance is abolished and the λ_{max} shifts to 340 nm, the absorption of the SLR oxime (Figure 3, curve C). Thus, the SLR analogue fulfills the commonly accepted criteria for ability to form a stable photosensitive rhodopsin pigment.

The spin-labeled retinal **2** synthesized to probe the bacteriorhodopsin retinal site[6] failed to form a pigment with bovine opsin. The only structural difference with the present analogue **3** is the double bond in the ring. The absence of the ring double bond in the present analogue must then release the ring from the requirement of coplanarity with the side chain containing the conjugated double-bond system, thus allowing the pyrrolidinyl ring to situate itself properly and fulfill the structural requirements of the binding site. Crouch and Or[2] proposed that the methyl groups on the chromophore are important in pigment formation as shown by the work using acyclic retinal analogues. The ability of the SLR ring to rotate about the single bond between the ring and side chain (related to the 6–7 bond in retinal) then allows two of the four methyl groups to occupy the crucial portions of the opsin binding site. Comparison of space-filling models of the SLR and retinal molecules indicates that the pyrrolidinyl ring of the SLR is about the same size as the cyclohexenyl ring of retinal and the methyl groups can be seen to approximate the position of those in retinal if the ring is properly rotated. Such rotation was not possible in the previous analogue **2**.

ESR of the SLR Pigment. The ESR spectrum of the 9-*cis*-SLR pigment in the dark approximates that of a "powder spectrum", the line shape that is characteristic of an immobilized spin-label with a rotational correlation time (τ_r) of greater than 10^{-7} s (Figure 2b).[16] The EPR spectrum is unchanged by the addition of NH_2OH or 11-*cis*-retinal. Additionally, incubation of the 9-*cis*-SLR with fully regenerated rhodopsin does not yield an immobilized ESR spectrum stable to NH_2OH. Illumination of the SLR pigment in the presence of NH_2OH leads to the abolition

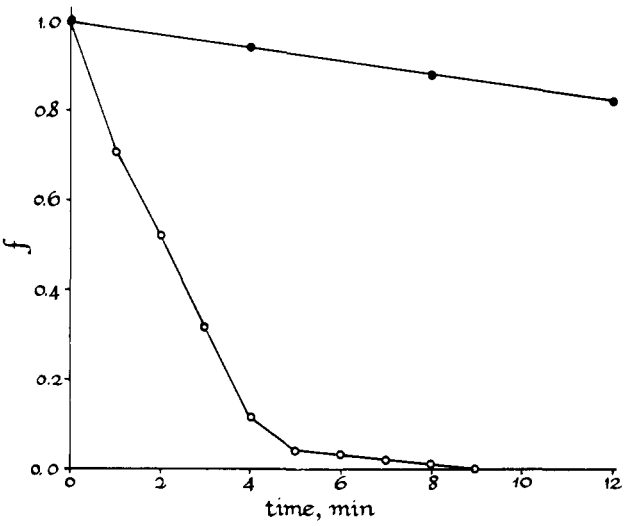

Figure 4. Comparison of rates of reduction of SLR in solution and SLR pigment by sodium ascorbate. Line height at time t divided by height at time t_0 is graphed vs. time t.

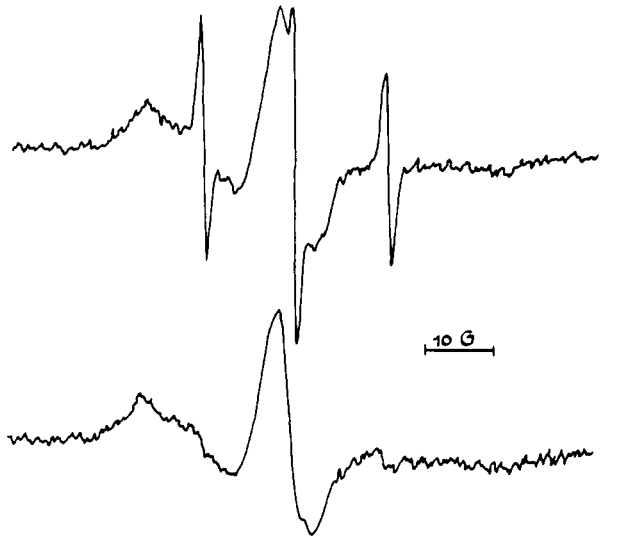

Figure 5. Effect of $NiCl_2$ on the ESR spectrum of SLR pigment: (top) SLR pigment, dark-adapted, in the presence of excess SLR in solution; (bottom) same, after addition of $NiCl_2$ (10 mM). Conditions: $a_0 = 16$ G; modulation amplitude 1.25 G; microwave power 7.5 mW; 22 °C.

of the bound signal, which then manifests as a free triplet spectrum identical with that of free SLR in solution. Thus, the behavior of the absorption spectrum is paralleled by that of the ESR spectrum under identical conditions, confirming that the SLR has formed a bonafide rhodopsin analogue and therefore reports on the microenvironment of the retinal binding site of the opsin protein.

Hydrophobicity of the Chromophore Binding Site. To characterize the degree to which the retinal binding site of rhodopsin excludes water and/or small hydrophobic molecules, the rate of reduction of sodium ascorbate in the presence of the SLR pigment suspension was observed. The rate at which a spin-label is able to be reduced by the ascorbate radical can be used as a measure of the availability of the spin-label to the aqueous environment.[17] If the label is in an aqueous environment, reduction will occur quickly. However, if the label is sequestered within a structure that excludes water, the reaction will occur at a much slower rate. The SLR pigment is essentially untouched by ascorbate, the ESR signal reducing only minimally, whereas the free spin-label reduces quickly (Figure 4). After 5 min, the free label was totally reduced

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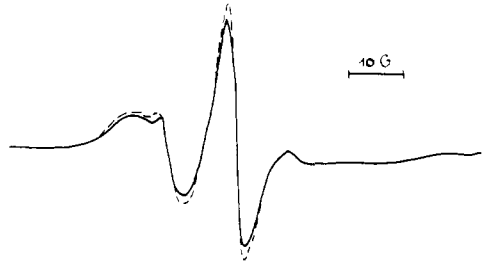

Figure 6. Orientation of ROS. SLR pigment film dried onto flat plate in vacuo. ESR spectra: (—) film oriented perpendicular to H_0; (---) film oriented parallel to H_0. Both recorded with film exposed to room air at 22 °C.

whereas the SLR pigment signal was less than 5% reduced. Similarly, the addition of the diamagnetic ion Ni^{2+}, which spin broadens the free nitroxide signal making it undetectable, was also used as a measure of aqueous sequestration,[18] and it was found that the SLR signal was unchanged at concentrations of Ni^{2+} that obliterated the signal of free spin-label (Figure 5).

Thus, the retinal binding site of bovine rhodopsin sequesters the ring end of the chromophore such that small water-soluble substances may not interact with the chromophore. Such hydrophobicity of the binding site is expected on the basis of the photophysics of the rhodopsin absorbance spectrum, which has its λ_{max} at wavelengths much longer than retinyl Schiff bases in solution.[19] The extensive electron delocalization is highly favored by a hydrophobic environment[20] that allows the local ionizable groups from the protein to dominate the modulation of wavelength of maximal absorbance. The protonated retinyl Schiff base of the pigment chromophore is accessible to water even in the dark adapted state;[21] however, the evidence of deGrip et al.[22] indicates that carboxyl groups in the region of the chromophore away from the Schiff base nitrogen are "buried" and do not interact with H_2O, in agreement with our data.

Orientation of the SLR. To determine whether the SLR is oriented in the membrane, a suspension of the SLR pigment was dried onto a flat plate to form oriented multilayers, and the ESR spectrum was recorded with the film exposed to room air so that the membranes were still minimally hydrated. This method yields highly oriented membranes with spin-labeled bacteriorhodopsin.[6] The ESR spectrum (Figure 6) resembled that of the membranes in suspension at the same temperature, showing the presence of at least two populations of spin-label. The spectrum was recorded with the plane of the film oriented perpendicular and parallel to the incident magnetic field. With this geometry, the axis of rotation of the rhodopsin protein lies in the direction of the magnetic field in both orientations. The shape and parameters of the spectrum do not depend on the orientation of the film to the magnetic field, which is expected on the basis of the known rotational mobility of the rhodopsin molecule in the ROS disk membrane.[23]

Temperature Dependence of the ESR Spectrum. The ESR spectra of the SLR pigment were recorded over the temperature range 0–45 °C. Over this range the outer hyperfine extrema were observed to retain their configuration and to maintain a separation of 64 G ($2A_{zz}$). As the temperature was raised, however, a second component was seen to appear and increase in intensity with temperature. This component was isolated by digital subtraction

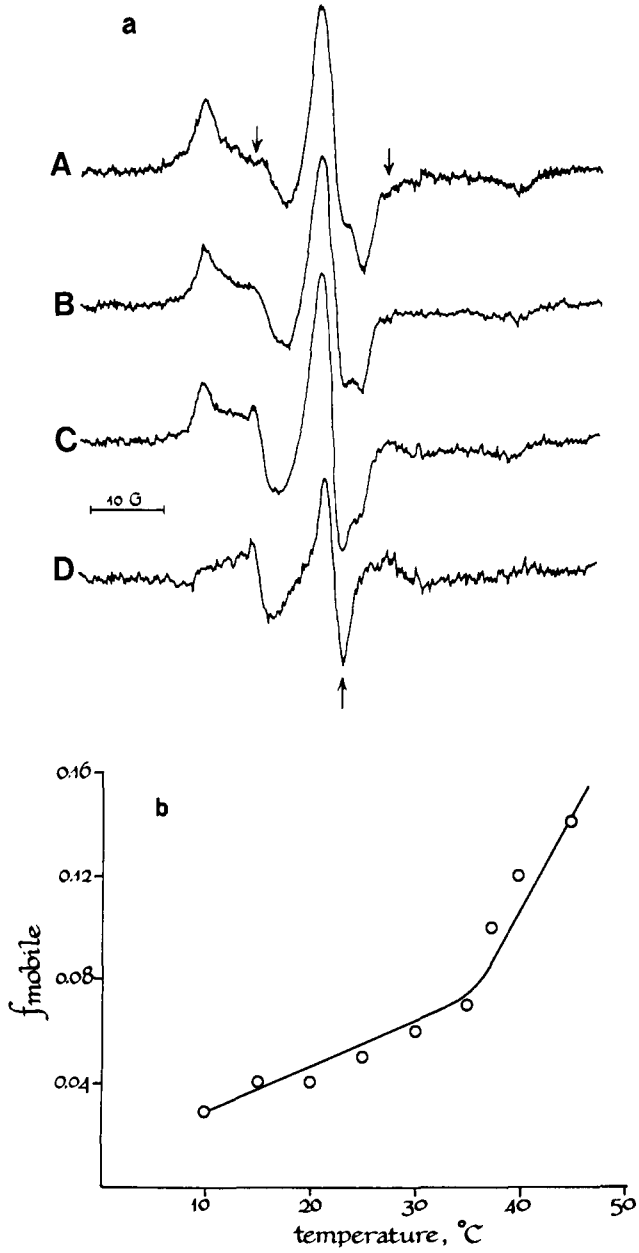

Figure 7. Effect of temperature on ESR spectrum of SLR pigment. (a) Curves A–C show ESR spectra at increasing temperature (0, 37, 45 °C). Curve D is obtained by digital subtraction of curve A from curve C. Arrows indicate resonance lines of fast component. (b) Fraction of SLR pigment molecules contributing to fast component of ESR spectrum graphed vs. temperature. $f = I_f/I_t$ where I_f and I_t are the double integrals of the fast component and total spectrum, respectively, obtained from digital subtraction of the fast component from complex spectra.

and can be seen to represent a subpopulation of SLR pigment that is rotating at a much faster rate than the homogeneous population at 0 °C (Figure 7a). The fraction of the fast component increases linearly with temperature, the curve showing a change in slope at 37 °C (Figure 7b). The line shape and outer hyperfine extrema ($2A_{zz}$ = 64.2 G) of the main component of the spectrum remain unchanged over this temperature range, and the effect is reversible.

This fast-moving component must represent a subpopulation of rhodopsins that exhibits either (1) increased rotational freedom or (2) a large reduction in the motional restriction imposed on the spin-label by the binding site. If the majority of rhodopsins were involved in the formation of stable oligomers, then a temperature-dependent increase in dissociated monomers could account for the observed results; however, there is evidence that rhodopsin does not form such stable complexes.[24] Kusumi et al.[25]

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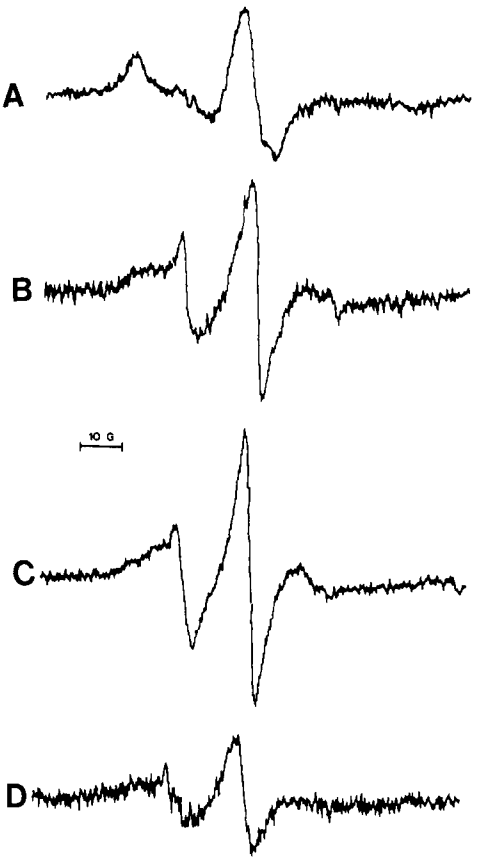

Figure 8. ESR spectra of SLR pigment in various detergents in 67 mM phosphate buffer, pH 7.4: (A) Digitonin (2%); (B) Triton X-100 (1%); (C) Ammonyx LO (1%); (D) CHAPS (1%). Parameters are as in Figure 2.

interpreted their saturation transfer EPR data in reconstituted systems as indicating that rhodopsin forms transient dimers ($\tau_r = 10^{-5}$ s) and that this associative behavior is temperature dependent in the physiological range of protein/lipid ratio. The effect on τ_r, and therefore on line shape, was to decrease it by a factor of 2 in the microsecond time scale, which theoretically should not be detected by the conventional ESR method used here. The large difference in magnitude of the change in τ_r for the subpopulation might reflect the differences between rhodopsin associative behavior in artificial membranes and that in the native disk membrane. Whether the effect observed here is related to the suggestion that there exist two kinetic forms of rhodopsin[26] is unclear.

The second possibility would require that the retinal binding site open up dramatically such that the pyrrolidinyl ring of the SLR could rapidly rotate about the 5–6 single bond, linking it to the isoprenoid side chain. We believe such a dramatic change in the binding-site structure in the dark would be unlikely and inconsistent with the finding that the SLR pigment is highly resistant to ascorbate reduction. However, in this system, there is a reversible temperature-dependent equilibrium between states of rhodopsin in the ROS membrane.

Detergent Effects. When the ESR spectra of the various detergent-solubilized pigments are compared to each other (Figure 8), it can be seen that the spectra vary widely depending on the detergent used. The spectra of the CHAPS and digitonin solubilized pigments more closely approximate that of the unsolubilized pigment in ROS suspension than those of the Triton X 100 or Ammonyx LO solubilized pigments. The strong nonionic detergent Triton X-100 led to significant denaturation of the SLR pigment, as both the UV–vis absorbance of the pigment and the immobilized component of the ESR spectrum were diminished on solubilization. Simiarly, solubilization in Ammonyx LO led to some destruction of the SLR pigment. Similar instability to stronger detergents has been noted in other rhodopsin analogue pigments as determined by changes in the visible absorption. Thus, these results show that stronger detergents affect the chromophore–protein interaction significantly more than the milder detergents and caution must be used when interpreting data obtained in detergent-solubilized systems in terms of physical properties of the binding site. This also points to obvious difficulties in comparing data on regenerated pigments between systems solubilized in different detergents.

Conclusion

A new spin-labeled retinal (SLR) has been synthesized as a probe of the retinal binding site of mammalian rhodopsin. A stable photosensitive isorhodopsin pigment is found having a maximum UV–vis absorbance consistent with other rhodopsin analog work. The ESR spectrum of the SLR pigment shows that the SLR is highly immobilized (τ_r greater than 10^{-7} s) and that the ring end of the chromophore is sequestered from the aqueous environment to the extent that it is inaccessible to small hydrophilic reagents. The ESR spectrum of the SLR pigment reflects the physiochemical characteristics of the retinal binding site and has shown structural differences between rhodopsin solubilized in different detergents. Thus the SLR molecule has been shown to be a sensitive and accurate probe of the chromophore binding site of mammalian rhodopsin. Further work with this pigment is currently underway to describe structural changes in the chromophore–protein interaction in the physiologically important metarhodopsin intermediates.

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