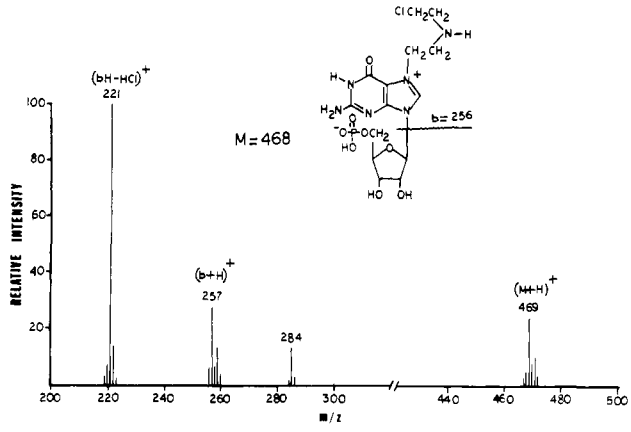

Figure 2. Positive-ion FAB mass spectrum of adduct II.

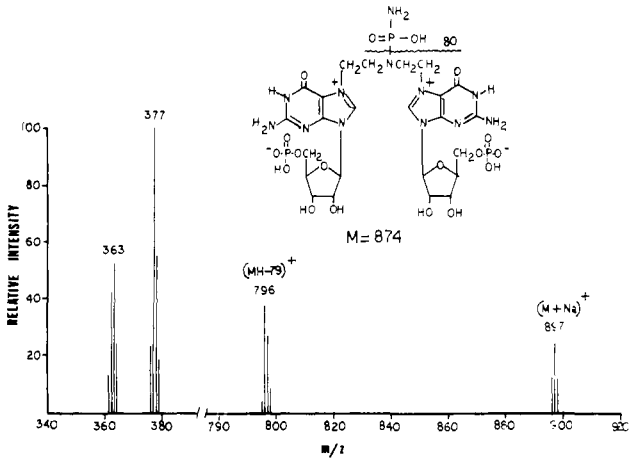

Figure 3. *Positive-ion FAB mass spectrum of adduct III.*

Adduct III produced an ion of mass 897 $(M + Na)^+$, which corresponds to the structure of a dimer adduct in which each arm of phosphoramide mustard has reacted with guanosine 5′-monophosphate at the 7-position (Figure 3). The fragment ion of mass 796 arises from the loss of the phosphorodiamidic group $(M + H - 79)^+$ as the result of N–P bond cleavage. Other peaks at *m/z* 363 and 377 probably represent the ions $(GMP + H)^+$ and $(7\text{-methyl-GMP})^+$.

We conclude that we have isolated three N-7-alkylated nucleotide adducts whose structures are shown in Figures 1–3. In agreement with a previous report,[6] we found guanosine nucleotide to be readily alkylated by phosphoramide mustard at the 7-position. Adduct II could arise either by scission of the N–P bond in phosphoramide mustard and subsequent alkylation of guanosine 5′-monophosphate by nornitrogen mustard or by alkylation of guanosine 5′-monophosphate by phosphoramide mustard and subsequent scission of the N–P bond.

Our study demonstrates that phosphoramide mustard, like other nitrogen mustards,[7,8] will alkylate N-7 of guanosine 5′-monophosphate. The cytotoxic effect of cyclophosphamide and phosphoramide mustard could be mediated by two major types of damage to DNA: depurination (deribosylation) of adducts I and II which could lead to chain scission and by interstrand and intrastrand cross-linking analogous to that of adduct III.

Acknowledgment. We are grateful to Dr. Ven L. Narayanan (NCI) for a generous gift of phosphoramide mustard. Mass spectral determinations were carried out at the Middle Atlantic Mass Spectrometry Laboratory, a National Science Foundation Regional Instrumentation Facility. NMR analyses were made by Dr. Lou Kan in the Johns Hopkins School of Hygiene. This work was supported by NIH grants GM-21248 and CA-16783 and training grant T32-CA-09243.

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A Bacteriorhodopsin Analogue Containing the Retinal Nitroxide Free Radical

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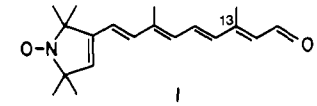
Synthetic analogues of retinal have been used to study the binding site and photochemistry of both the visual pigment rhodopsin[1] and the bacteriorhodopsin[2] membrane of *Halobacterium halobium*. Nitroxides are excellent "reporter" groups for studying protein environments by the use of electron spin resonance (ESR) spectroscopy. A retinal derivative which contains a nitroxide functional group as well as forms a pigment with the bleached purple membrane or with the visual pigment apoprotein, opsin, would indeed be a valuable probe of the binding site. We have reported the synthesis of one spin-labeled retinal and the pigment analogue formed between it and the bleached purple membrane.[3] The information which could be gained from this analogue was limited as the label was linked to the retinal molecule by an ester bond which hydrolyzed readily after pigment formation. We report here the synthesis of the nitroxide retinal **1**, which contains the *N*-oxide incorporated into the ring of the retinal. The properties of the stable pigment formed from the reaction of the 13-cis or all-trans isomers of **1** and the bleached purple membrane are discussed.



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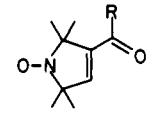
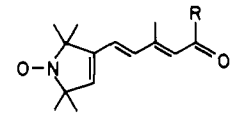


2a, R = OH
2b, R = H



3a, R = OC_2H_5
3b, R = H

Esterification of 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid (Eastman) (**2a**) with diazomethane followed by lithium aluminum hydride reduction and manganese dioxide oxidation, both in dry tetrahydrofuran, afforded the corresponding aldehyde **2b**[4] (44%). Condensation of **2b** with triethyl 4-phosphono-3-methylcrotonate[5] in the presence of sodium amide gave the ester **3a** (77%) which was reduced and oxidized as above to yield the aldehyde **3b** (39%) which was purified by preparative thin-layer chromatography (TLC). Anal. Calcd for $C_{14}H_{20}NO_2$: C, 71.75; H, 8.62; N, 5.98. Found: C, 71.59; H, 8.70; N, 6.01. The aldehyde **3b** was condensed, reduced, and oxidized as above to yield the spin-labeled retinal **1** in 41% yield. The product was purified by TLC and high-pressure liquid chromatography (HPLC) using 20% ethyl acetate/hexane on a μ-Porasil column. Two major isomers were obtained and identified as the 13-cis and trans isomers by elution profile on HPLC (13.5 and 15.2 min, respectively) and pigment formation capability. The mass spectrum of the two isomers each had a parent peak at *m/e* 300 (70 eV).

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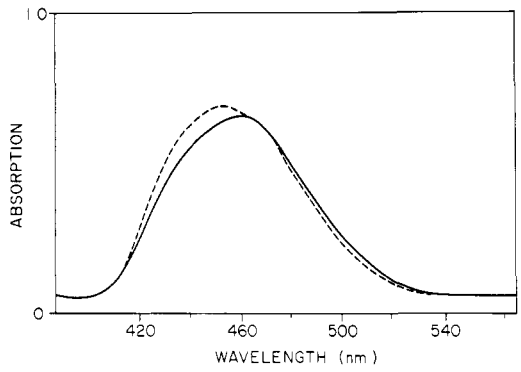

Figure 1. Absorption spectra of *N*-oxide bacteriorhodopsin in water. (—) Light adapted; (-h-) dark adapted. Dark-adapted sample irradiated (slide projector, 400 W, glass sharp cut-off Corning 2A filter) for 5 min at room temperature.

Anal. Calcd for $C_{19}H_{26}NO_2$: C, 75.95; H, 8.74; N, 4.66. Found: C, 76.09; H, 8.60; N, 4.80. The 13-cis and trans isomers absorbed at 375 and 374 nm, respectively. The infrared spectrum (ethanol) showed the presence of the aldehyde C=O (1724 cm^{-1}) and the N–O (960 cm^{-1}). ESR spectrum of the spin-labeled retinal in 1% ethanol/water showed the characteristic nitroxide three-line pattern with *g* value of 2.034 and hyperfine splitting of 16.3 G. By assuming the extinction coefficient is the same as that of all-*trans*-retinal and calculating the concentration of nitroxide groups with a standard compound, 4-hydroxyl-1-oxyl-2,2,6,6-tetramethylpiperdine, one unpaired electron was found per molecule. The correlation time of this retinal *N*-oxide was estimated to be 3.1×10^{-9} s.[6]

Spin-labeled pigment was regenerated from bleached purple membrane[7] and the 13-cis or all-trans spin-labeled retinal by the procedure previously described;[8] the pigments from both isomers appeared to be identical. The absorption maximum of the pigment (454 nm) is considerably blue shifted from the native pigment (528 nm) and red shifts to 459 nm on light adaptation (Figure 1). We have observed blue absorption maximum shifts for other artificial pigments substituted with electronegative groups in the 4 position on retinal;[3] such a blue shift from the native chromophore is predicted by the model for determining absorption maximum that places an external point charge close to the ring end of retinal.[3,9]

The spin-labeled pigment is stable to the addition of hydroxylamine (0.1 M, for 60 min). *trans*-Retinal does not displace the spin-labeled chromophore from the binding site. Therefore, the retinal derivative **1** is bound to the same lysine as is retinal in the native pigment.

Absorbance changes in light- and dark-adapted samples after brief flashes were measured with a kinetic spectrophotometer.[10] Measurements were made at 380, 440, 490, 500, and 510 nm with broad-band actinic light flashes of complementary wavelengths. Interestingly, no absorbance changes that could be associated with the photocycle of the light-adapted pigment could be detected. A small absorbance decrease and increase was observed at 440 and 510 nm, respectively. The half-decay time of these absorption changes was on the order of several seconds, which would be consistent with the changes expected when part of the pigment goes from dark-adapted to light-adapted state. There was flash-induced absorbance increase at 380 nm, but it was not correlated in either time or magnitude with changes in the pigment's main absorption band. The origin of this transient change is as yet unexplained. In addition, the dye *p*-nitrophenol was used to try to detect light-induced proton release or uptake from the spin-labeled pigment containing the membrane sheet;[10] no proton changes were observed.

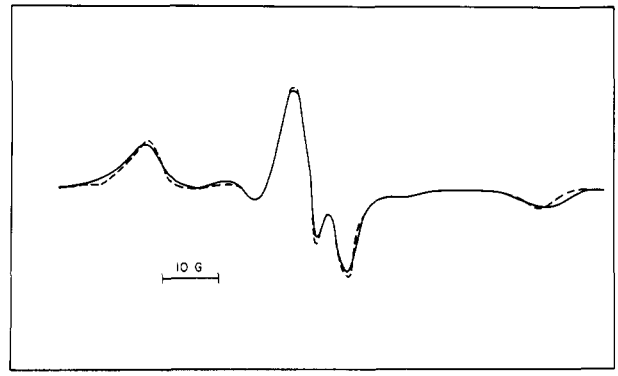

Figure 2. ESR spectra of light- and dark-adapted *N*-oxide bacteriorhodopsin analogue in water at 15 °C. (—) Light adapted; (---) dark adapted. Sample in flat cell irradiated for 5 min in spectrometer cavity with light through a 415-nm sharp cut off filter (Corning). Time constant, 1.0 s; gain, 2000; scan time, 8 min; modulation amplitude, 2.0 G; microwave power, 7 mW.

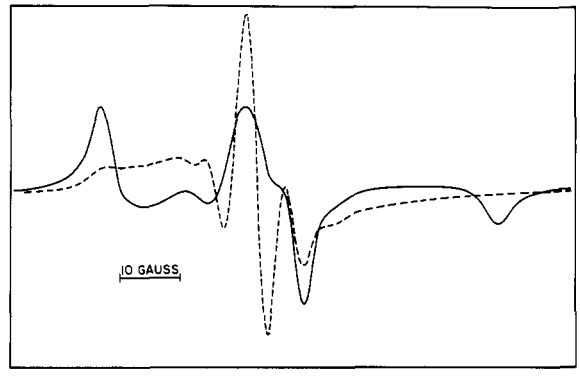

Figure 3. ESR spectra of *N*-oxide bacteriorhodpsin analogue in a dry film at 20 °C. (—) Film parallel to magnetic field; (---) film perpendicular to magnetic field. Time constant, 1.0 s; scan time, 8 min; modulation amplitude, 8.0 G; gain, 2000; microwave power, 7 mW.

The ESR spectrum of the pigment shows an anisotropic signal characteristic of a near-rigid limit nitroxide spectrum[11] with a calculated correlation time of $\sim 10^{-7}$ s (Figure 2). When the dark-adapted pigment was irradiated in the ESR cavity with light through a 415-nm sharp cut off filter, a slight increase in A_z[12] and Δ_L[12] were observed, indicating there may be an increase in the fluidity of the protein environment in the vicinity of the chromophore ring (Figure 2). For this pigment no phase transition in the range of 0–70 °C was detected as no changes in the ESR signal were observed in this range.

The addition of sodium ascorbate to the retinal *N*-oxide in 1% ethanol/water results in complete reduction of the nitroxide within 5 min as demonstrated by the loss of the isotropic ESR signal. In contrast the pigment *N*-oxide is resistant to reduction since several hours are required for the anisotropic signal to be eliminated by the ascorbate.

Dried films of the pigment demonstrated a high degree of angular dependence (Figure 3). A 64-G separation was observed between the hyperfine extrema in the spectrum obtained with the film parallel to the magnetic field. This result is indicative of lack of rotation of the probe about any axis.[13] No difference was observed between the dried and hydrated films.

In conclusion, a stable spin-labeled bacteriorhodopsin analogue has been formed. The absorption maximum of the pigment is blue

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shifted from that of the native pigment, probably due to the interaction of the electronegative N>O group with the negative counterion which is proposed to be in the region of the chromophore ring.[9] The ESR spectra indicate that the environment of the chromophore ring has a high degree of orientation and is rigid within the membrane although a slight increase in mobility occurs on dark adaptation.

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Theoretical Studies of the 1,2-Hydrogen Shift. 11. The Controversial Barrier Height between Silaethylene and Methylsilylene

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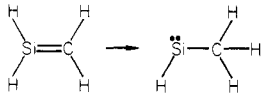
Received August 13, 1981

Earlier papers in this series[1-10] have emphasized that for one of the simplest reaction types, the 1,2-hydrogen shift, theory is now able to make constructive additions and amendments to what is known from experiment. For example, our research and that of Pople and co-workers[11-13] have demonstrated that the singlet rearrangements of hydrocarbon diradicals such as methylcarbene ($CH_3\ddot{C}H$) and vinylidene ($CH_2{=}C{:}$) occur with little or no barrier. In distinct contrast, the analogous triplet rearrangements face substantive barriers, e.g., ~40 kcal for the methylnitrene ($CH_3\ddot{N}\cdot$) rearrangement to methylenimine.[6] Thus a number of general trends are beginning to appear in this area.

A particularly controversial problem lying within the scope of the 1,2-hydrogen shift is the magnitude of the barrier between silaethylene and methylsilylene



(1)

In an earlier theoretical study, Goddard, Yoshioka, and Schaefer (GYS)[7] predicted this barrier to be 41 kcal at the unlinked cluster corrected configuration interaction (CI) level of theory employing a standard double-ζ (DZ) basis set.[14] The examination of analogous studies performed at a higher level of theory would appear to suggest[8-13] that extension of the basis set, treatment of higher order correlation effects, and correction for zero-point vibrational energies would all reduce the predicted barrier, but not to less than 25 kcal.

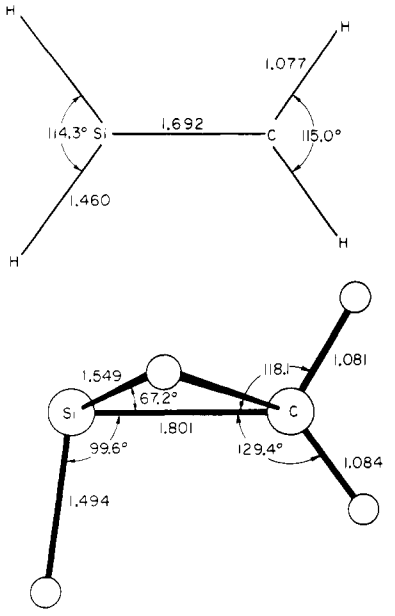
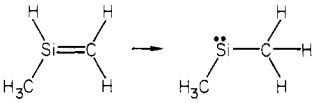


Figure 1. Theoretical DZ + P SCF stationary-point geometries for silaethylene and the transition state connecting it with methylsilylene. Bond distances are in angstroms.

In light of this theoretical background, the publication of dissenting back-to-back experimental communications[15,16] on this topic came as something of a surprise to us. Conlin and Wood[15] reported evidence that the reaction of 1-methylsilaethylene to dimethylsilylene



(2)

is rapid. Specifically they point to pyrolysis of methylsilacyclobutane leading to the isolation of products characteristic of dimethylsilylene reactions. Moreover, Conlin and Wood suggested that the isomerization (1) of the parent silaethylene might be even more rapid than (2).

In the second communication, Drahnak, Michl, and West (DMW)[16] present matrix isolation results which suggest that reaction 2 proceeds rapidly at 100 K, and the product dimethylsilylene is then trapped. DMW cite the previously discussed GYS theoretical study[7] but conclude that "unless the additional methyl [i.e., the difference between reactions 1 and 2] has a dramatic effect, this (theoretical) result is not compatible with our interpretation. No simple alternatives have occurred to us." Thus there would appear to be a conflict between the theoretical prediction[7] that the barrier is not less than ~25 kcal and the experimental deduction that this same barrier is perhaps 5 kcal or less.

In light of this apparent discrepancy between theory and experiment, it was deemed imperative to reexamine the barrier for reaction 1 at a higher level of theory. Specifically, it was thought that the addition of polarization basis functions might significantly alter the earlier predictions.[7] Therefore a set of p functions (orbitals exponent $\alpha = 1.0$) was added to each of the four hydrogen

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