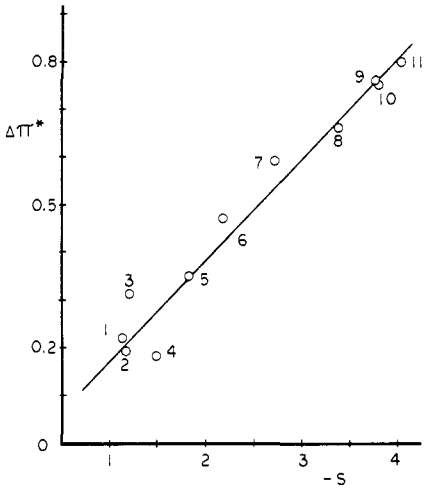

Figure 1. $\Delta\pi^*$ ($\pi^*_{\text{polar solute}} - \pi^*_{\text{model solute}}$) vs. the coefficient s, where $\pi^*(\text{solutes}) = -0.0122 - 0.2031s$. The points represent (1) Et_3N ($\pi^* = 0.14$)/Et_3CH (−0.08);[7] (2) anisole (0.73)/toluene (0.54); (3) Bu_2O (0.24)/octane (−0.08);[7] (4) pyridine (0.87)/benzene (0.59); (5) Et_2O (0.27)/butane (−0.08);[7] (6) nitrobenzene (1.01)/toluene (0.54); (7) benzene (0.59)/cyclohexane (0.00); (8) 5-nonanone (0.58)[7]/nonane (−0.08);[7] (9) butanone (0.67)/butane (−0.08);[7] (10) cyclohexanone (0.67)/cyclohexane (0.00); (11) acetone (0.72)/propane (−0.08).[7]

and ΔH_v (or ΔH_{subl}) of the solutes need not be known. We have correlated ΔH_p values (eq 2, typical uncertainty ±0.1 kcal/mol) in 14 solvents (1,2-dichloroethane, carbon tetrachloride, *tert*-butyl alcohol, methanol, DMF, Me_2SO, triethylamine, benzene, toluene, mesitylene, *n*-butyl ether, ethyl acetate, cyclohexane, and heptane) for a variety of ethers, ketones, and other dipolar and polarizable solutes, with π^* of the solvents (kcal/mol):

ΔH_p(anisole vs. toluene) = $-1.332 - 1.160\pi^*$, r = 0.925, sd = 0.109

ΔH_p(Et_3N vs. Et_3CH) = $0.115 - 1.130\pi^*$, r = 0.971, sd = 0.086

ΔH_p(Bu_2O vs. octane) = $-0.781 - 1.202\pi^*$, r = 0.821, sd = 0.199

ΔH_p(pyridine vs. benzene) = $-0.580 - 1.485\pi^*$, r = 0.952, sd = 0.124

ΔH_p(Et_2O vs. butane) = $-0.912 - 1.813\pi^*$, r = 0.895, sd = 0.223

ΔH_p(nitrobenzene vs. toluene) = $-2.470 - 2.174\pi^*$, r = 0.899, sd = 0.273

ΔH_p(benzene vs. *c*-C_6H_{12}) = $0.514 - 2.718\pi^*$, r = 0.962, sd = 0.174

ΔH_p(5-nonanone vs. nonane) = $-1.113 - 3.378\pi^*$, r= 0.961, sd = 0.280

ΔH_p(cyclohexanone vs. *c*-C_6H_{12}) = $-1.544 - 3.756\pi^*$, r = 0.957, sd = 0.296

ΔH_p(butanone vs. butane) = $-1.380 - 3.895\pi^*$, r = 0.900, sd = 0.383

ΔH_p(acetone vs. propane) = $1.386 - 4.030\pi^*$, r = 0.959, sd = 0.308

The numerical coefficient of π^*(solvents) (s) tends to increase with the "polarity" of the solute, but the most dipolar solute, nitrobenzene (greatest μ), and the least dipolar, benzene, have similar values of s (Figure 1). The correlation with s becomes very good if the measure of "polarity" is taken to be $\Delta\pi^*$, where

$$\Delta\pi^* = \pi^*(\text{polar solute}) - \pi^*(\text{model solute}) \quad (4)$$

This is appropriate because the model compounds are not equally "nonpolar" (noninteractive).

If the s coefficients for the above relationships are correlated with $\Delta\pi^*$, the relationship is

$$\Delta\pi^*(\text{solutes}) = -0.0122 - 0.2031s,\ r = 0.978,\ \text{sd} = 0.035,\ n = 11 \quad (5)$$

For a polar solute in a series of solvents the second term in eq 3, $[\Delta H_s(\text{polar solute}) - \Delta H_s(\text{model solute})]_{\text{ref solvent}}$, is a constant, so

$$[\Delta H_s(\text{polar solute}) - \Delta H_s(\text{model solute})]_{\text{polar solvent}} = \Delta H_p - k \quad (6)$$

Correlation of the left-hand side of eq 6 with π^*(solvents) yields a different intercept than does eq 3, but the value of s is the same. One can therefore determine ΔH_s of a solid polar compound and the model compound in a series of solvents, correlate the difference in ΔH_s with the π^* values of the solvents, and obtain the value of s. Equation 5 provides the value of $\Delta\pi^*$(solutes) corresponding with that value of s. If a value of π^* of the (usually hydrocarbon) model is known or can be reasonably estimated, π^* of the polar solute can be calculated (eq 4).

Acknowledgment. We thank Dr. Mortimer J. Kamlet for helpful discussions. This study was supported by the Robert A. Welch Foundation (Grant E-136).

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(7) Estimated values of π^*. All alkanes have been assigned the value (−0.08) found experimentally for hexane and heptane.[2] π^* for ketones[2] decreases with increasing size and hindrance to the carbonyl group. A value slightly smaller than that for 3-heptanone (0.59) has been estimated for 5-nonanone (0.58).

Photoaffinity Labeling of Bacteriorhodopsin with 3-([1-^{14}C]Diazoacetoxy)-*trans*-retinal

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Purple membrane[1] is a light-energy transducer that uses bacteriorhodopsin to pump protons across the cell membrane[2] to generate ATP. Bacteriorhodopsin folds into seven α-helical segments spanning the cell membrane.[3] The primary sequence of the apoprotein (opsin)[4,5] and the attachment site of the chromophore (retinal) to lysine-216[6-8] through a protonated Schiff base linkage[9,10] have been established.

Bacteriorhodopson (bR) has two modifications:[2] (i) the light-adapted form (bR^{LA}), λ_{max} 570 nm, containing *trans*-retinal, which is responsible for the proton pump inducing photocycle; and

(1) Oesterhelt, D.; Stoeckenius, W. *Nature (London), New Biol.* **1971**, *233*, 149-152.
(2) "Methods in Enzymology"; Packer, L., Ed.; Academic Press: New York, London, 1982; Vol. 88, Biomembranes, Part I, Visual Pigments and Purple Membranes II.
(3) Unwin, P. N. T.; Henderson, R. *Nature (London)* **1975**, *257*, 28-32.
(4) Ovchinnikov, Yu. A.; Abdulaev, N. G.; Feigina, M. Yu.; Kiselev, A. V.; Lobanov, N. A. *FEBS Lett.* **1979**, *100*, 219-224.
(5) Khorana, H. G.; Gerber, G. E.; Herlihy, W. C.; Gray, C. P.; Anderegg, R. J.; Nihei, K.; Biemann, K. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 5046-5050.
(6) Bayley, H.; Huang, K.-S.; Radhakrishnan, R.; Ross, A. H.; Takagaki, Y.; Khorana, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 2225-2229.
(7) Lemke, H.-D.; Oesterhelt, D. *FEBS Lett.* **1981**, *128*, 255-260.
(8) Mullen, E.; Johnson, A. H. Akhtar, M. *FEBS Lett.* **1981**, *130*, 187-193.
(9) (a) Lewis, A.; Spoonhower, J.; Bogomolni, R. A.; Lozier, R. H.; Stoeckenius, W. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 4462-4466. (b) Aton, B.; Doukas, A. G.; Callender, R. H.; Becher, B.; Ebrey, T. G. *Biochemistry* **1977**, *16*, 2995-2999.
(10) (a) Rothschild, K. J.; Marrero, H. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 4045-4049. (b) Bagley, K.; Dollinger, G.; Eisenstein, L.; Singh, A. K.; Zimanyi, L. *Ibid.* **1982**, *79*, 4972-4976.

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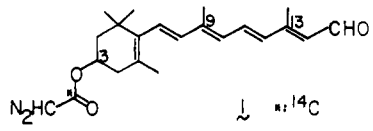
(ii) the dark-adapted form (bR^{DA}), λ_{max} 560 nm, consisting of a 1:1 mixture of *trans*- and 13-*cis*-retinals,[11] which is not involved in the proton pumping.[12] We have recently secured the first experimental evidence that the 13-trans/cis isomerization in bR^{LA} is probably essential for proton translocation.[13] An external point charge model[14,15] was proposed to account for the purple color of bR; furthermore, a working model for the tertiary structure of bR has been forwarded.[16] The present studies were initiated to assist in clarifying the bR structure and eventually the mechanism of proton pumping.

Chromophore. Studies on 3-(diazoacetoxy)-9-*cis*-retinal and bovine opsin had shown that the diazoacetate group, which absorbs at 245 nm, is well suited for photoaffinity labeling.[17] The trans isomer **1** was therefore synthesized by a route similar to that



previously employed[17] and purified by HPLC to give the racemic mixture at C-3: UV (*n*-hexane) 360 nm (ϵ 49 000) and 245 nm (ϵ 18 000, diazoacetoxy); FTIR (film) 2140 (diazo), 1690 (ester), and 1658 cm^{-1} (aldehyde); ^1H NMR spectrum[18] ascertained trans geometry of the polyene chain and also showed a signal at 4.74 ppm due to the diazoacetoxy methine proton.

3-([1-^{14}C]Diazoacetoxy)-*trans*-retinal **1** was prepared by reaction of [1-^{14}C]glyoxylic acid tosylhydrazone[19] with 3-hydroxy-*trans*-retinal.[20] After purification by flash column chromatography and HPLC (LiChrosorb Si 60, 10 × 250 mm, 12% ethyl acetate/*n*-hexane) the compound had a specific activity of 1.4 μCi/μM. The structural integrity of the radioactive material was checked by HPLC (μ-Porasil, 3.9 × 300 mm, 20% ether/*n*-hexane), which showed the activity to be exactly halved when coinjected with an equal amount of cold material.

Formation of Light-Adapted and Dark-Adapted Pigments. 3-(Diazoacetoxy)-bR was prepared by addition of 1 OD[21] of **1** (cold) in 10 μL of EtOH to a suspension of 1 OD[21] of bleached purple membrane[22] in 1 mL of 10 mM Hepes buffer, pH 7.0, and stirring in the dark for 1–2 h. The light-adapted species thus formed[12] absorbed at 532 nm;[23] longer periods of incubation, e.g., 15 h,[24] resulted in conversion to the dark-adapted bR absorbing at 525 nm,[25] which had a shoulder due to contamination around 440 nm. The slight excess of chromophore and the contaminant were removed by treatment with bovine serum albumin[26] to give the purified pigment, which when submitted to the CH_2Cl_2 procedure[27] showed only the presence of trans and 13-cis chromophores, 3:1 ratio. The radioactive (diazoacetoxy)-bR purified similarly showed a count of 43 000 cpm/OD.

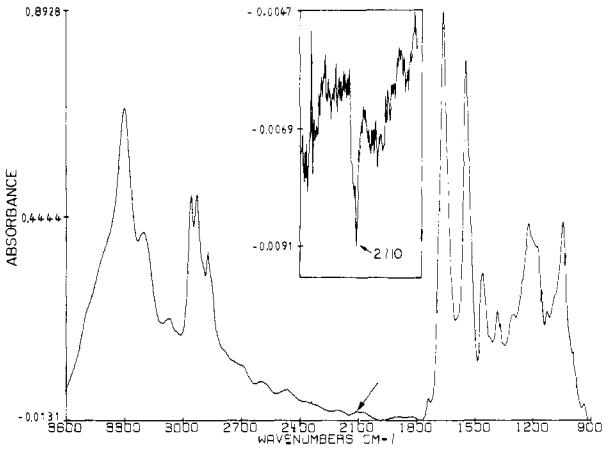


Figure 1. FTIR of 3-(diazoacetoxy)bR^{DA}. Insert is difference FTIR after and before 254-nm irradiation. Spectra were measured as a hydrated film of ca. 2 OD of pigment deposited on an IRTRAN plate (see ref 10b); the photoaffinity label was directly activated on the plate.

The bR^{DA} could be light adapted to 532 nm bR^{LA} by a 10-min irradiation at room temperature with a 1000-W light of >530 nm; this in turn was dark adapted to the 525-nm species after standing 6 h in the dark. The 532-nm bR^{LA} was stable to irradiation with light of >530 nm; however, irradiation in the presence of NH_2OH led to formation of retinal oxime and bleaching of pigment. These properties as well as the interconversion of light- and dark-adapted species are very similar to natural bR. The 532-nm bR^{LA} showed a biphasic CD at ca. 505 nm (+)/590 nm (−) characteristics of the trimeric structure of bR.[28] Addition of *trans*-retinal did not displace the 3-(diazoacetoxy)retinal from the binding site.

The light-adapted pigment was incorporated into soybean phospholipid vesicles and proton translocation was assayed according to published methods.[29] Irradiation at >530 nm resulted in alkalinization of the medium, indicating proton uptake by the vesicles. The amount of protons pumped was ca. 50% of that of regenerated bR^{LA} assayed under the same conditions. The ability of the retinal analogue to induce light-driven proton translocation provides further support for occupancy of the natural binding site and adequacy of the bR analogue for photoaffinity studies.

The optimal condition to maximize chromophore cross-linking while minimizing pigment destruction was a 4-h irradiation at 4 °C with a 4-W Hg lamp combined with a 254-nm narrow-band interference filter. This led to considerable reduction in CH_2Cl_2 extractable (non-cross-linked) chromophore and only ca. 10%

(11) (a) Pettei, M. J.; Yudd, A. P.; Nakanishi, K.; Henselman, R.; Stoeckenius, W. *Biochemistry* **1977**, *16*, 1955–1959. (b) Maeda, A.; Iwasa, T.; Yoshizawa, T. *J. Biochem.* **1977**, *82*, 1599–1604. (c) Sperling, W.; Rafferty, C. N.; Kohl, K.-D.; Dencher, N. A. *FEBS Lett.* **1979**, *97*, 129–132.

(12) The role of bR^{DA} is not known. Note that when *trans*-retinal is incubated in the *dark* with opsin it gives the *light*-adapted bR (chromophore is trans), which upon 40-min standing in the dark converts to bR^{DA} (trans:13-cis = ca. 1:1); irradiation with 570-nm light regenerates bR^{LA}, which pumps protons. Incubation of pure 13-cis isomer in the dark gives a pigment absorbing at 548 nm.

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(16) Engelman, D. M.; Henderson, R.; McLachlan, A. D.; Wallace, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2023–2027.

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(18) ^1H NMR ($CDCl_3$, 250 MHz) δ 1.13 (s, 1-Me), 1.09 (s, 1-Me), 1.74 (s, 5-Me), 2.04 (s, 9-Me), 2.35 (d, J = 0.73 Hz, 13-Me), 4.74 (s, diazoacetate CH), 5.16 (m, 3-H), 5.99 (d, J = 8.4 Hz, 14-H), 6.15 (d, J = 16.1 Hz, 8-H), 6.21 (d, J = 12.1 Hz, 10-H), 6.29 (d, J = 16 Hz, 7-H), 6.39 (d, J = 15 Hz, 12-H), 7.15 (dd, J = 12.1, 15 Hz, 11-H), 10.12 (d, J = 8.05 Hz, 15-H).

(19) Blankley, C. J., Sauter, F. J.; House, H. O. *Org. Synth.* **1969**, *49*, 22–27.

(20) 3-Hydroxy-*trans*-retinal was synthesized from 3-hydroxy-β-ionone by a two-carbon and a five-carbon elongation sequence according to a procedure similar to that described previously[17] for the synthesis of the 9-cis isomer.

(21) 1 OD of retinal **1** is the amount of retinal giving OD = 1 at 360 nm when dissolved in 1 mL of hexane and measured in a 1-cm pathlength cell; 1 OD of bleached membrane/mL is the amount of opsin adjusted to regenerate OD = 1 at 570 nm (bR^{LA}) in a 1-cm pathlength cell.

(22) Oesterhelt, D.; Stoeckenius, W. *Methods Enzymol.* **1974**, *31*, 667–678.

(23) It is likely that in the case of the photoaffinity-labeled visual pigments, one of the 3-enantiomers binds preferentially. This aspect is currently under study.

(24) Formation of bR^{DA} depends on the sample preparation, in some cases the time being less than 2 h.

(25) Incubation of the 3-(diazoacetoxy)-13-*cis*-retinal in the dark yields a pigment absorbing at 500 nm.

(26) BSA has been used to remove retinal oxime from bleached purple membranes. This is accomplished by treating the pigment with a 2% solution of fatty acid free bovine serum albumin (BSA) in pH 7.4 buffer followed by water washes to remove BSA (Katre, N. V.; Wolber, P. K.; Stoeckenius, W.; Stroud, V. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4068–4072).

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reduction in the 532-nm pigment band intensity (photolysis of 3-(diazoacetoxy)retinal in *n*-hexane under this condition led to total disappearance of its 245-nm band).

The extent of cross-linking, i.e., ca. 25%, was estimated by taking aliquots at suitable intervals during irradiation, denaturing the pigment by heating in SDS for 2-3 min, adding EtOH, and scintillation counting the pellet obtained by centrifugation.[30]

An advantage of the diazoacetoxy photoaffinity group is that its characteristic IR frequency around 2150 cm^{-1} is in a region normally transparent in biopolymers. Thus although the diazo band is too weak to be observed in the FTIR of the pigment prior to cross-linking (Figure 1, arrow), the difference spectrum measured after irradiation at 254 nm clearly shows the 2110-cm^{-1} band due to disappearance of the photoaffinity group (Figure 1, insert).[31] Studies are in progress to locate the site(s) of labeling in bR.[32]

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Registry No. 1, 78324-68-2; [^{14}C]-**1**, 86309-92-4; 3-hydroxy-*trans*-retinal, 6890-91-1; [1-^{14}C]glyoxylic acid tosylhydrazone, 86309-93-5.

(30) We thank Drs. H. Bayley and K.-S. Huang for this procedure (to be published).

(31) The difference in frequencies of the diazo group in the unbound chromophore (2140 cm^{-1}) and bound chromophore (2110 cm^{-1}) is presumably due to environmental effects.

(32) Collaboration with Prof. H. G. Khorana and co-workers.

Evidence for the Necessity of Double Bond (13-Ene) Isomerization in the Proton Pumping of Bacteriorhodopsin

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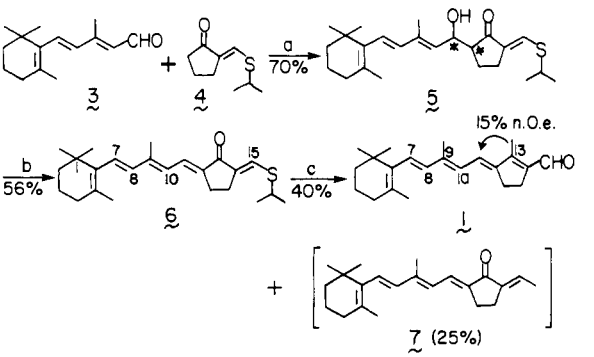
Bacteriorhodopsin (bR), the pigment of purple membrane (PM), converts solar energy into a proton gradient that is coupled to ATP synthesis.[1] bR consists of a protein (opsin) that binds one retinal molecule at Lys-216[2] through a protonated Schiff base linkage.[3,4] There are two modifications for bR,[5] the light- and dark-adapted forms, bR^{LA} (570 nm) and bR^{DA} (560 nm), the chromophores of which are *trans*-retinal and 1:1 mixture of *trans*- and 13-*cis*-retinals.[6] Although both forms undergo a photocycle, only that of bR^{LA} is associated with H^+ pumping.

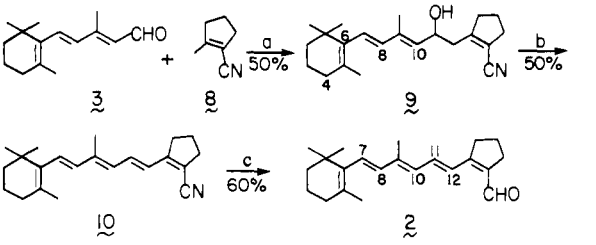
Proton translocation during the photocycle is thought to be associated with changes in the protonation state of the Schiff base linkage as well as retinal geometry. However, the structures of photocycle intermediates, e.g., M_{412} species, and their relation to the mechanism of proton pumping is not clear. Although resonance Raman and FTIR spectroscopy[3b,8a,b,d] have shown that the M_{412} species is not protonated, results pertaining to the nature of 13-ene in M_{412} are conflicting, i.e., it is a 1:1 mixture of cis/trans,[6a] 13-trans,[8b] or mostly 13-cis.[3b,7,8a,c] Therefore information pertinent to the molecular events involved in the proton pumping was sought by the study of retinals **1** and **2** with fixed 13-trans and 13-cis structures. The bR analogues derived from these retinals both *failed to pump protons, thus showing that the 13-ene isomerization appears to be necessary for proton translocation.*

Scheme I



[a] (i) **4** in LDA/THF −78 °C, 15 min; (ii) **3**, −78 °C, 20 min; (iii) 3 equiv of AcOH, −78 °C. [b] (i) $MsCl/Et_3N/CH_2Cl_2$, 0 °C, 1 h; (ii) flash chromatography. [c] (i) $MeLi/Et_2O$, −78 °C; (ii) satd aq NH_4Cl, −30 °C → 25 °C (30 min); (iii) flash chromatography.

Scheme II



[a] (i) LDA/THF, −78 °C → 25 °C (40 min); (ii) 2 equiv of HMPA, 0 °C; (iii) addition of **3**, −78 °C (1 h) → 0 °C (40 min). [b] (i) Ac_2O/py, 25 °C, 2 h; (ii) *t*-BuOK/THF, 0 °C, 30 min. [c] (i) DIBAL/Et_2O, −78 °C (1 h) → −40 °C; (ii) EtOAc, −40 °C, followed by aq $(COOH)_2$, −40 °C (15 min) → 25 °C.

The trans-fixed aldehyde **1** was synthesized according to Scheme I. The C_{15}-aldehyde **3** was condensed in aprotic medium with thiovinyl ketone **4** (from 2-(hydroxymethylidene)cyclopentanone[9] and 2-propanethiol,[10] mild conditions[11]) to give β-hydroxy ketone **5** as a 55:45 diastereomeric mixture (^{1}H NMR).[12] Dehydration of **5** with $MsCl/NEt_3$[13] provided thiovinyl ketone **6** as the major product: mp 130.5-132.0 °C (hexane); UV (hexane) 386 nm.[14]

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(2) (a) Bayley, H.; Huang, K.-S.; Radhakrishnan, R.; Ross, A. H.; Takagaki, Y.; Khorana, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 5046-5050. (b) Lemke, H. D.; Oesterhelt, D. *FEBS Lett.* **1981**, *128*, 255-260. (c) Mullen, E.; Johnson, A. H.; Akhtar, M. *Ibid.* **1981**, *130*, 187-193.

(3) (a) Lewis, A.; Spoonhower, J.; Bogomolni, R. A.; Lozier, R. H.; Stoeckenius, W. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 4462-4466. (b) Aton, B.; Doukas, A. G.; Callender, R. H.; Becher, B.; Ebrey, T. G. *Biochemistry* **1977**, *16*, 2995-2999.

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(9) Thompson, W. C. *J. Am. Chem. Soc.* **1931**, *53*, 3160-3164.

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