lation of the cage radical ions results in electronically excited-state generation.[17] One explanation for the observed 10% singlet yield is that the decarboxylation and charge annihilation are competitive with spin equilibration. Thus, the dioxetanones that react by the CIEEL path, but do not generate an excited singlet activator, may be generating the undetected triplet excited state of the hydrocarbon. The final step in the sequence is light emission from the excited activator, which we detect as chemiluminescence.

In competition with the CIEEL path, uncatalyzed unimolecular decomposition of the dioxetanone generates electronically excited acetone. The combination of these two excitation mechanisms accounts for all of the experimental observations on the chemiluminescence of dioxetanone **1**.

In summary, we have shown that an efficient CIEEL pathway is the major light generating process from dioxetanone **1** with any one of several easily oxidized activators. This is the third documented example of efficient chemiluminescence by this route.[4,18] We are continuing our investigation of the chemiluminescence of dioxetanones to further establish the details of the mechanism in this case. We are also investigating other chemiluminescent systems that appear to react by the CIEEL path.

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References and Notes

(1) These results were presented in part at the 174th National Meeting of the American Chemical Society, Chicago, Ill., Aug 28, 1977.
(2) For leading references, see T. Wilson, *Int. Rev. Sci.: Phys. Chem., Ser. Two*, **9**, 265 (1976).
(3) E. H. White, J. D. Miano, and M. Umbreit, *J. Am. Chem. Soc.*, **97**, 198 (1975); O. Shimomura, T. Goto, and F. H. Johnson, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 2799 (1977). See, however, F. I. Tsuji, M. DeLucca, P. D. Boyer, S. Endo, and M. Atukegawa, *Biochem. Biophys. Res. Commun.*, **74**, 606 (1977).
(4) J.-y. Koo and G. B. Schuster, *J. Am. Chem. Soc.*, **99**, 6107 (1977).
(5) This point has been discussed more fully: J.-y. Koo, S. P. Schmidt, and G. B. Schuster, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
(6) W. Adam and O. Cueto, *J. Org. Chem.*, **42**, 38 (1977); W. Adam and J.-C. Liu, *J. Am. Chem. Soc.*, **94**, 2894 (1972); W. Adam, A. Alzerreca, J.-C. Liu, and F. Yany, *J. Am. Chem. Soc.*, **99**, 5768 (1977).
(7) Typical conditions: [1] = 10^{-4} M in CH_2Cl_2 at 24.5 °C. Light was detected by the photon counting technique. Spectral resolution was achieved with a Jarrel-Ash 0.25 M monochromator.
(8) W. Adam, G. A. Simpson, and F. Yany, *J. Phys. Chem.*, **78**, 2559 (1974).
(9) The intensity was determined at 2×10^{-5} M in activator in CH_2Cl_2 solution at 24.5 °C under low resolution conditions so that the emission spectra were Gaussian. The intensities were corrected for photomultiplier tube and monochromator efficiency according to data supplied by the manufacturers, and also for a small contribution by singlet–singlet energy transfer from directly generated acetone singlet according to

$$I_{chl} = k_2[\text{Act}][\mathbf{1}]\phi_{\text{CIEEL}} + k_1[\mathbf{1}]\phi_{ET}^{SS} \cdot \phi_S^S$$

where I_{chl} is the chemiluminescence intensity, ϕ_{CIEEL} is the efficiency of production of excited singlet activator by the induced decomposition, ϕ_{SS}^{ET} is the efficiency of energy transfer from acetone singlet to the activator, and ϕ_S^S is the efficiency of unimolecular acetone singlet generation. All solutions contained 20 μL of 5% aqueous Na_4EDTA to suppress metal catalyzed reactions.
(10) The yield of light was determined relative to tetramethyldioxetane (TMD) using 9-10-dibromoanthracene as the acceptor. The yield of acetone triplet was taken to be 30%,[11a] the triplet–singlet energy transfer efficiency 25%,[11] and the fluorescence quantum yield 10%.
(11) (a) T. Wilson, D. E. Golan, M. S. Harris, and A. L. Baumstark, *J. Am. Chem. Soc.*, **98**, 1086 (1976); (b) V. M. Berenfel'd, E. V. Chumavskii, M. P. Grinev, Yu. I. Kuryatnikov, E. T. Artem'ev, and R. V. Dzhagatspanyan, *Bull. Acad. Sci. USSR, Phys. Ser. (Eng. Transl.)*, **3**, 597 (1970).
(12) Firefly bioluminescence is claimed to be 80% efficient. W. D. McElroy, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, **19**, 941 (1960).
(13) For example see J. K. Kochi and H. E. Mains, *J. Org. Chem.*, **30**, 1862 (1965).
(14) J.-y. Koo and G. B. Schuster, unpublished results.
(15) Peroxy esters typically have reduction potentials of ca. −0.9 eV while diacyl peroxides reduce at ca. −0.1 eV vs. the saturated calomel electrode: L. S. Silbert, L. P. Witnauer, D. Swern, and C. Ricciati, *J. Am. Chem. Soc.*, **81**, 3244 (1959). Simple alkyl peroxides reduce at ca. −1.6 eV: E. J. Kuta and F. W. Quackenbush, *Anal. Chem.*, **32**, 1069 (1960).
(16) Air-saturated solutions were nearly as efficient as argon-purged solutions. Free radical anions should react rapidly with O_2.
(17) L. R. Faulkner, *Int. Rev. Sci.: Phys. Chem., Ser. Two*, **9**, 213 (1977).
(18) J. P. Smith and G. B. Schuster, *J. Am. Chem. Soc.*, in press.
(19) Fellow of the Alfred P. Sloan Foundation, 1977–1979.

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Molecular Recognition of DNA by Small Molecules. Synthesis of Bis(methidium)spermine, a DNA Polyintercalating Molecule

Sir:

The molecular recognition of DNA by small molecules is an important macromolecular receptor–drug interaction in the field of chemotherapy.[1] The formation of noncovalently bound nucleic acid–drug complexes produces profound pharmacological effects by interfering with biological processes in which nucleic acids participate.[1,2] The fact that DNA is a *defined macromolecular receptor* allows a rational approach to drug design and permits a unique opportunity for studying site-specific drug-binding processes. The therapeutic importance and the possibility for a detailed chemical understanding of the mechanism of action of these DNA–drug complexes provide sufficient stimulus to develop a rational methodology for optimizing the thermodynamics, kinetics, sequence specificity, structural specificity, and chemical specificity of these binding ligands.[3]

Some ligands that bind noncovalently to duplex DNA do so by a process called intercalation, the insertion of a flat molecule between the base pairs of a double helix.[4] Typical binding constants for these complexes are $K \sim 10^5$ M^{-1}.[5] We report here initial findings in our laboratories directed at increasing the binding affinity of drugs to nucleic acid by the synthesis of polyintercalating agents.[6] We describe the synthesis and study of a new molecule, bis(methidium)spermine (**1**, BMSp) and provide supporting evidence that (1) BMSp is a double intercalator, (2) has a binding site size of four base pairs, and (3) binds at least 10^4 times stronger to DNA than the simple monomer.

We chose to study the dimer of a well-characterized intercalating molecule the antitrypanosomal agent and nucleic acid probe, ethidium bromide **2** ($K \sim 10^5$ M^{-1}).[7] The synthetic strategy employed here preserves the major structural attributes of the ethidium monomer as an intercalator. The tetramine, spermine, was chosen to link the intercalators because of its known affinity for nucleic acid[8] and its length which allows a geometry sufficient to reach nonadjacent intercalation sites in accordance with the neighbor exclusion binding mode[9] (see Figure 1). For a bisintercalated species this polyamine connector should lie intimately in the groove of the DNA helix. Structural modifications of any linker with respect to charge, chirality, length, flexibility, and functionality are expected to play an important part in controlling the stability and nature of these polyintercalator–DNA complexes.

The synthetic sequence is outlined in Scheme I. Nitration of *o*-aminobiphenyl (**3**) (potassium nitrate/sulfuric acid),[10] condensation with *p*-cyanobenzoyl chloride, and cyclization (phosphorous oxychloride) yielded 6-(4-cyanophenyl)-3,8-dinitrophenanthridine. Successive methylation (dimethyl sulfate), hydrolysis, and reduction (reduced iron powder/HCl) afforded maroon crystals of 5-methyl-6-(4-carboxylphenyl)-3,8-diaminophenanthridinium chloride monochloride monohydrate (*p*-carboxylmethidium chloride, **4**) in an overall yield of 16%. The infrared and NMR spectra of compound **4** were

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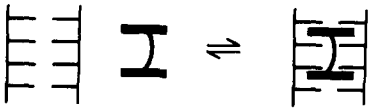


Figure 1.

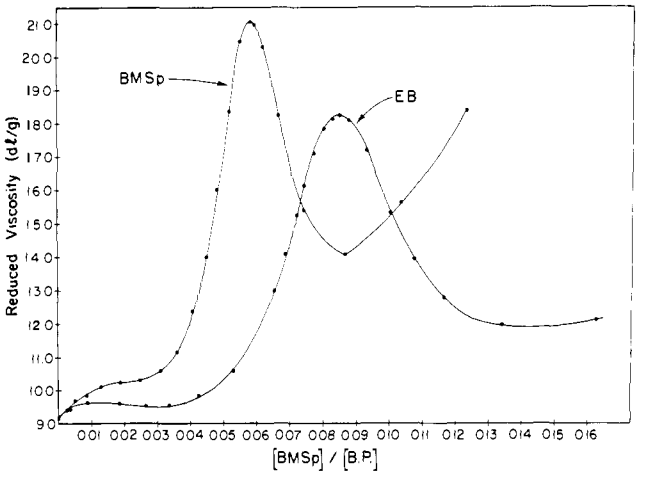
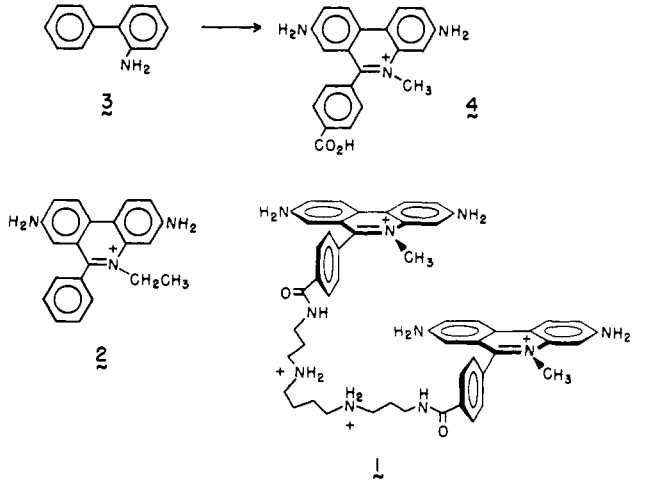


Figure 2. Viscometric titration of closed circular PM2 DNA with BMSp (upper curve) and ethidium bromide (lower curve).

Scheme I



identical with those of an authentic sample.[11] The reaction of 0.5 equiv of spermine with the acylimidazole ester of **4** in dry Me_2SO for 24 h at 25 °C afforded, upon concentration, a purple solid.[12] This solid was chromatographed in 100-mg portions on 500 g of silica gel 60 (70–230 mesh ASTM) using 0.5% HCl/methanol[13] as the elution solvent (70% yield). Rechromatography of the maroon crystals yielded analytically pure solid bis(methidium)spermine hydrochloride **1**.[14]

It is known that binding of ethidium bromide (EB) **2** removes and reverses the supercoiling of closed circular DNA owing to local unwinding of the helix resulting from the intercalation event.[15] To test for bisintercalation the viscometric titrations[16,19] of superhelical PM2 DNA with BMSp and EB were carried out (Figure 2). From the observed drug/base pair ratios at the maximum of the titration and the known unwinding angle of EB (26°),[17] the unwinding angle of BMSp is calculated to be 38.4 ± 0.2°.[18] Since the value of the unwinding angle of BMSp is only 1.5 times the unwinding of EB, the observed unwinding angle could reflect equal contributions from mono- and bisintercalated species. However, if at these drug/base pair ratios (BMSp/BP ≤ 0.06) there is no monointercalation component, these data require that the intercalating chromophores do not intercalate independently of one

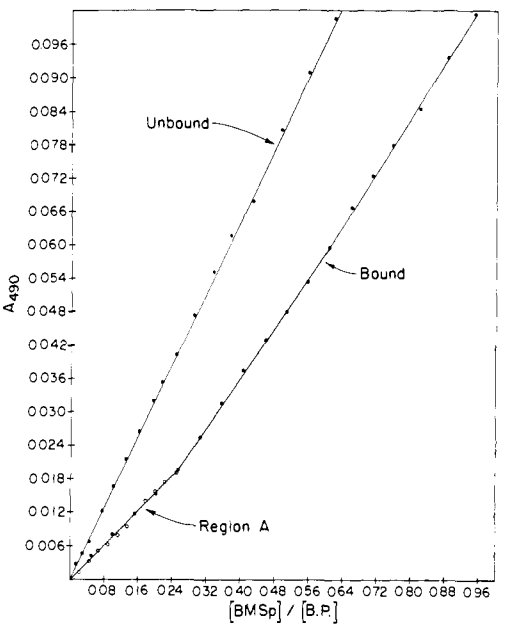


Figure 3. Spectrophotometric titration of calf thymus DNA with BMSp. The concentration of DNA was 1.726×10^{-6} M in base pairs (BP). The results of two separate titrations are shown for region A (BMSp/BP ≤ 0.25).

another and the resulting bisintercalation geometry is not identical with the geometry assumed by two independent ethidium molecules.[20]

The binding of BMSp to calf thymus DNA has been monitored by absorption and fluorescence spectroscopy because, like ethidium, a metachromic shift[22] and quantum yield increase[23] result when BMSp binds to DNA. The binding of BMSp to calf thymus DNA as monitored by absorption spectroscopy at 490 nm, a wavelength where the extinction coefficients of bound and unbound BMSp differ most, is shown in Figure 3.[24] The change in absorbance (ΔA_{490}) is linear from a BMSp/base pair ratio (BMSp/BP) of 0 to 0.25 (region A). After BMSp/BP = 0.25 there is a sharp break in the observed ΔA_{490}.[25] The observation that the slope in this region is greater than that exhibited by unbound BMSp[26] but less than that exhibited in region A reflects the appearance of (an) *additional* bound BMSp species distinct from those formed in region A.

The binding of BMSp to calf thymus DNA as monitored by fluorescence spectroscopy is shown in Figure 4.[27] The increase in the fluorescence of BMSp in the presence of DNA (I_1) minus the fluorescence of an equivalent solution of BMSp in the absence of DNA (I_0) is plotted against the ratio BMSp/BP.[23] We find in agreement with the spectrophotometric titration, that there are at *least two bound forms* of BMSp. A highly fluorescent complex is formed for BMSp/BP ratios of 0 to 0.25 and the fluorescence of this species is quenched by additional bound forms for BMSp/BP ratios > 0.25.[28]

We assign the bound species observed for BMSp/BP ratios of 0 to 0.25 to a bisintercalated species based on the observed binding stoichiometry, and spectral properties of this species. The metachromic shift is identical in magnitude with the metachromic shift exhibited by ethidium,[22] reflecting intercalation of both chromophores of BMSp into the DNA helix. The observed ratio of quantum yields of bound (ϕ_B) and unbound BMSp (ϕ_F), $\phi_B/\phi_F \simeq 41$,[29] is also consistent with bisintercalation based on the known mechanism of fluorescent enhancement of ethidium by DNA.[30] Moreover, the observed stoichiometry of one BMSp per four base pairs is identical with the stoichiometry that would be predicted for a bisintercalated species based on the known stoichiometry of ethidium (one

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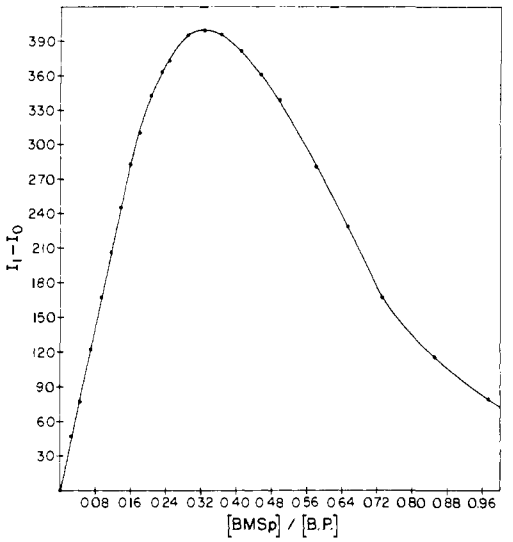

Figure 4. Fluorescence titration of calf thymus DNA with BMSp. The concentration of DNA was 1.726×10^{-6} M in base pairs.

ethidium/two BP)[31] and in accordance with the nearest neighbor exclusion model.[9]

The calculation of the binding affinity of bisintercalated BMSp from the fluorescence and spectrophotometric titration data requires a knowledge of the dependence of the spectral and fluorescence properties of the bisintercalated species as a function of the degree of saturation and the effect of other bound BMSp species on the quantum yield of the bisintercalated species. These uncertainties plus the inability to detect sufficient unbound BMSp renders the traditional Scatchard analysis unreliable. Nevertheless, a *minimum* binding constant can be estimated which is compatible with both the fluorescence and spectrophotometric titration data. We find that the binding constant of bisintercalated BMSp is $\geq 4 \times 10^9$ M^{-1},[32] which can be compared with 3×10^5 M^{-1} [35] for ethidium (EB) under similar conditions.

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References and Notes

(1) (a) J. W. Corcoran Ed., and F. E. Hahn, Ed,. "Mechanism of Action of Antimicrobial and Antitumor Agents", Springer-Verlag Heidelberg, 1975; (b) E. F. Gale, E. Cundliffe, and P. E. Reynolds, "The Molecular Basis of Antibiotic Action", Wiley, London, 1972: (c) T. A. Connors and J. J. Roberts, Ed., "Platinum Coordination Compounds in Cancer Chemotherapy", Springer-Verlag, Heidelberg, 1974.
(2) (a) B. Fridlender and A. Weissbach, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 3116 (1971); (b) J. P. Richardson, *J. Mol. Biol.*, **78**, 703 (1973).
(3) For example, in the case of ethidium bromide, see (a) J. L. Bresloff and D. M. Crothers, *J. Mol. Biol.*, **95**, 103 (1975); (b) ref 5a; (c) D. J. Patel and L. L. Canuel, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 3343 (1976); (d) J. L. Bresloff and D. M. Crothers, unpublished work; (e) T. R. Krugh, F. N. Wittlin, S. P. Cramer *Biopolymers*, **14**, 197 (1975).
(4) L. S. Lerman, *J. Mol. Biol.*, **3**, 18 (1961).
(5) (a) F. Quadrifoglio, V. Crescenzi, and V. Giancotti, *Biophys. Chem.*, **1**, 319 (1974); (b) F. Quadrifoglio and V. Crescenzi, *ibid.*, **2**, 64 (1974).
(6) (a) J. B. LePecq, M. L. Bret, J. Barbet, and B. Rogues, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 2915 (1975); (b) M. J. Waring, *Biochem. J.*, **157**, 721 (1976); (c) E. S. Canellakis, Y. H. Shaw, W. E. Hanners, and R. A. Schwartz, *Biochim. Biophys. Acta*, **418**, 277 (1976); (d) R. M. Fico, T. K. Chen, G. S. Canellakis, *Science*, **198**, 53 (1977).
(7) See M. J. Waring in ref 1a.
(8) (a) S. Z. Hirschman, M. Leng, and G. Felsenfeld, *Biopolymers*, **5**, 227 (1967); (b) L. Stevens, *Biol. Rev.*, **45**, 1 (1970).
(9) (a) D. M. Crothers, *Biopolymers*, **6**, 575 (1968); (b) see ref 15b; (c) P. J. Bond, R. Langridge, K. W. Jennette, and S. J. Lippard, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 4825 (1975).
(10) J. Cymerman and W. F. Short, *J. Chem. Soc.*, No. 1, 703 (1949).
(11) Supplied by May and Baker Ltd, Nottingham, England.
(12) The acylimidazde ester of **4** was prepared by stirring for 1 h 1 equiv each of **4**, *N*-ethylmorpholine, and acyldiimidazole in Me_2SO at 25 °C.
(13) L. F. Fieser and M. Fieser, "Reagents for Organic Synthesis", Vol. 1, Wiley, New York, N.Y., 1967, p 688.
(14) (a) BMSp was isolated as the trihydrochloride salt. Anal. Calcd: C, 60.32; H, 5.94; N, 13.53; Cl 17.12. Found: C, 60.07; H, 5.76; N, 13.41; Cl, 16.82. The infrared spectrum (KBr) exhibited both amide (1630, 1540 cm^{-1}) and methylene (2950, 1425 cm^{-1}) absorptions associated with the incorporation of spermine. (b) At physiological pH BMSp exists as the tetracation.
(15) (a) J. Vinograd and W. Bauer, *J. Mol. Biol.*, **33**, 141 (1968); (b) C. C. Tsai, S. C. Jain, and H. M. Sobell, *ibid.*, **114**, 301 (1977).
(16) B. Revet, M. Schmir, and J. Vinograd, *Nature New Biol.*, **229**, 10 (1971).
(17) J. C. Wang, *J. Mol. Biol.*, **89**, 783 (1974).
(18) Based on two separate determinations. The extinction coefficient of ethidium bromide was taken at 480 nm to be 5700 M^{-1} cm^{-1} (average of several literature values).
(19) J. M. Saucier, B. Festy, and J. B. LePecq, *Biochimie*, **53**, 973 (1971).
(20) Previous studies[21] have shown that substitution of an ethyl for a methyl group or addition of a *p*-carboxyl group to ethidium bromide has little effect on the unwinding angle.
(21) M. J. Waring and L. P. Wakelin, *Mol. Pharmacol.*, **9**, 544 (1974).
(22) M. J. Waring, *J. Mol. Biol.*, **13**, 269 (1965).
(23) J. B. LePecq and C. Paoletti, *J. Mol. Biol.*, **27**, 87 (1967).
(24) Spectrophotometric titrations were conducted in H_2O–phosphate buffer (pH 6.9, 0.025 M $KHPO_4$, 0.025 M Na_2PO_4) at 25 °C in 10-cm cells on a Carey 14 spectrophotometer using the 0→0.1, 0.1→0.2 slidewire.
(25) The observation of a sharp break in ΔA_{490} was found to be independent of wavelength over the range monitored (360–700 nm).
(26) The A_{490} for unbound BMSp as a function of [BMSp] is reexpressed in terms of [BMSp]/[BP] by dividing [BMSp] by the [BP] used in determining A_{490} for bound BMSp.
(27) Fluorescence titrations were conducted in D_2O (>99%)–phosphate buffer (0.01 mM EDTA, 0.025 M $KHPO_4$, and 0.025 M Na_2PO_4) at 25 °C on a Perkin-Elmer MPF-4 fluorimeter. Excitation was at 482 nm and emission monitored at 640 nm.
(28) We have observed that the quenching of fluorescence which takes place for BMSp/BP > 0.25 at these low ionic strengths is substantially reduced when the ionic strength is increased. Similar behavior has been observed for ethidium suggesting that at least one of the additional bound BMSp species is an electrostatically bound nonintercalated species.[23]
(29) Uncorrected for polarization.
(30) J. Olmstead and D. Kearns, *Biochemistry*, **16**, 3647 (1977).
(31) W. Bauer and J. Vinograd, *J. Mol. Biol.*, **47**, 419 (1970).
(32) Assuming that the extinction coefficient and quantum yield of bisintercalated BMSp are independent of the degree of saturation, the percent bound BMSp at BMSp/BP = 0.25 is found to be ≥97% and equal to 88% by quantitative analysis of the spectrophotometric and fluorescence data, respectively.[23] Since the quantum yield of ethidium does depend on the degree of saturation[33] and since we observe quenching at high BMSp/BP ratios, a minimum estimate of the percent bound can be calculated by assuming that the discrepancy between the spectrophotometric and fluorescence data arises only from a change in the extinction coefficient of bound BMSp. If the extinction coefficient of bound BMSp at BMSp/BP = 0.25 is assumed to decrease 7% of its original value at high BP/BMSp, then both spectrophotometric and fluorescence titrations yield the same percent bound (92%) at BMSp/BP = 0.25. This estimate corresponds to a minimum binding constant of $\geq 4 \times 10^9$ M^{-1} in terms of the von Hippel–McGhee model.[34]
(33) J. Bontemps and E. Frederick, *Biophys. Chem.*, **2**, 1 (1974).
(34) J. D. McGhee and P. H. von Hippel, *J. Mol. Biol.*, **86**, 469 (1974).
(35) The binding constant of ethidium for a monovalent cation concentration of 0.075 M was obtained by linear extrapolation of previously published data.[23] The resulting binding constant was then corrected for nearest neighbor exclusion by the model of von Hippel–McGhee.[34]
(36) Alfred P. Sloan Research Fellow, 1977–1979.
(37) National Institutes of Health Trainee (GM-01262).

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Influence of Alkyl Substitution on the Trans → Cis Photoisomerization of *all-trans*-Retinal and Related Polyenes

Sir:

Although the primary photochemical step and subsequent sequence of chemical events following absorption of a photon of light by the visual protein rhodopsin is uncertain,[1-8] it is known that the 11-*cis*-retinyl chromophore[1] undergoes a cis → trans isomerization[2,3] forming *all-trans*-retinal (structure **1**) and the protein opsin as the final products[4] of the rhodopsin bleaching process. To better understand the nature of the factors that may influence the photochemically initiated transformation of the chromophore in rhodopsin, we have examined the solution photochemical properties of the isomeric retinals[9,10] and related synthetic polyenes. The photochemical

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