Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 12273–12277, December 1994
Biophysics

Chemiluminescent activation of the antiviral activity of hypericin: A molecular flashlight

(retrovirus/energy transfer/photosensitized chemotherapy)

S. CARPENTER*†, M. J. FEHR‡, G. A. KRAUS‡, AND J. W. PETRICH†‡

Departments of *Microbiology, Immunology, and Preventive Medicine, and ‡Chemistry, Iowa State University, Ames, IA 50011

Communicated by John D. Corbett, August 22, 1994 (received for review April 25, 1994)

ABSTRACT **Hypericin is a naturally occurring photosensitizer that displays potent antiviral activity in the presence of light. The absence of light in many regions of the body may preclude the use of hypericin and other photosensitizers as therapeutic compounds for the treatment of viral infections *in vivo*. The chemiluminescent oxidation of luciferin by the luciferase from the North American firefly *Photinus pyralis* was found to generate sufficiently intense and long-lived emission to induce antiviral activity of hypericin. Light-induced virucidal activity of hypericin was demonstrated against equine infectious anemia virus, a lentivirus structurally, genetically, and antigenically related to the human immunodeficiency virus. The implications for exploiting chemiluminescence as a "molecular flashlight" for effecting photodynamic therapy against virus-infected cells and tumor cells are discussed.**

The need for effective antiviral therapies for treatment of human immunodeficiency virus (HIV) infections has acquired increasing urgency with the realization that there may be years before an effective vaccine is in widespread use (1). Three compounds currently are approved for use in treatment of HIV-1 infections, and all target the viral enzyme reverse transcriptase (2). The eventual emergence of drug-resistant viral variants likely contributes to the fact that the present therapies may delay, but do not completely block, the progression to clinical disease in HIV-1-infected persons. Consequently, attention currently is focused on the development of combination therapies that employ a variety of compounds targeting different stages in the virus life cycle. A promising candidate is hypericin (Fig. 1), a naturally occurring polycyclic quinone that displays potent light-induced antiviral activity against a number of enveloped viruses, including HIV-1 (3–7).

Hypericin is a photosensitizing compound (8). The antiviral activity of hypericin is enhanced >100-fold in the presence of light (3–7). Upon illumination, hypericin produces singlet oxygen very efficiently, with a quantum yield of 0.73 (9); some studies have suggested that its antiviral activity is due to the production of singlet oxygen (4–6). The excited-state reactivity of hypericin, however, extends well beyond the photosensitization of oxygen to form singlet oxygen. Recent work suggests that antiviral activity may be due to complex mechanisms involving the superoxide anion and hypericinium ion, implicating a type I radical mechanism (10–12). Moreover, we have observed that oxygen is not required for the antiviral activity of hypericin (13).

The mechanism by which hypericin inactivates HIV infectivity is not clear. Meruelo and coworkers (4, 5) have reported that, in the presence of light, hypericin induces significant changes in the HIV capsid protein p24; they suggest that cross-linking and other alterations of p24 may inhibit the release of reverse transcriptase activity. It is significant that these workers found that under ambient lighting conditions, 4 μM hypericin did not inhibit the binding of gp120 to CD4 cells or the formation of syncytia. However, inhibition of gp120 binding was observed under conditions of more intense illumination (4). Other studies also have reported inhibition of syncytia formation under relatively high levels of illumination (6). Together, these results suggest that observed differences in the biological effects of photoactivated hypericin depend on the irradiance and the concentration of photosensitizer. Thus, under relatively low light conditions, there is minimal damage to viral and/or cell membranes and the antiviral activity is associated with changes in viral capsid proteins. With increasing light intensity, the biological effects expand to include interactions between virus and cell membranes.

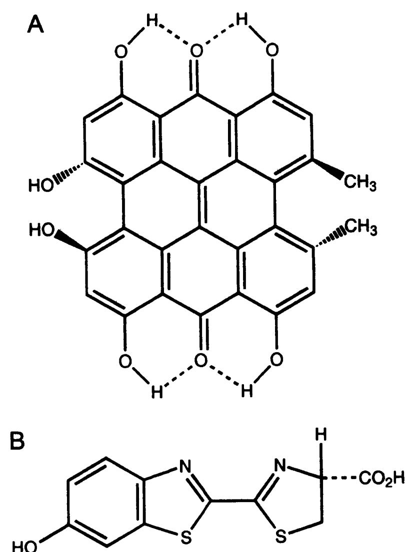

FIG. 1. Structures of hypericin (*A*) and luciferin (*B*).

The usefulness of photosensitizers such as hypericin for treatment of viral infections *in vivo* is hampered by the dependence on light for optimal virucidal activity. In this article, we discuss a strategy to place in the proximity of hypericin a chemiluminescent light source so that photodynamic therapy can be extended to all regions of the body. What is required is a light source that emits a broad band of wavelengths in the region where the photoactive molecule absorbs. An excellent choice for the light source is luciferin (Fig. 1). The reaction of luciferin with the enzyme luciferase and molecular oxygen produces light in the 520- to 680-nm region with a quantum efficiency of about unity (14–18) (Fig.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HIV, human immunodeficiency virus; EIAV, equine infectious anemia virus; FFU, focus-forming units.
†To whom reprint requests should be addressed.

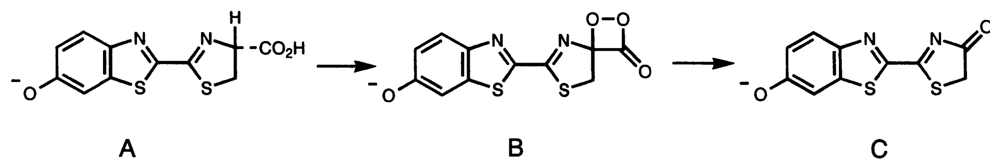

FIG. 2. Crucial intermediates in the production of firefly chemiluminescence (14–19). Luciferin (*A*) is catalyzed by the enzyme luciferase in the presence of ATP, Mg^{2+}, and O_2 to form the high energy four-member peroxide or dioxetanone intermediate (*B*). This intermediate subsequently decarboxylates to form the chemiluminescent species oxyluciferin (*C*).

2). Hypericin absorbs light strongly in this range (Fig. 3), suggesting that energy transfer between the product of the chemiluminescent reaction (Fig. 2) and hypericin may be sufficient to effect significant antiviral activity.

MATERIALS AND METHODS

Reagents. Hypericin was obtained from Carl Roth (Karlsruhe, Germany) and solubilized in dimethyl sulfoxide to 1 mg/ml. Stock solutions were stored at 4°C. Luciferase from the North American firefly (*Photinus pyralis*) and luciferin were obtained from Sigma and resuspended in glycylglycine buffer (25 mM glycylglycine/15 mM $MgSO_4$/4 mM EGTA, pH 7.8); aliquots were stored at −60°C.

Optical Measurements. For optical assays, luciferase and luciferin were resuspended in glycylglycine buffer and reactions were initiated by the addition of a freshly prepared solution of ATP. Light output was measured with a liquid nitrogen-cooled charge-coupled device (Princeton Instruments LN/CCD-1152UV; Trenton, NJ) mounted on an HR320 (Instruments SA, Edison, NJ) monochromator with a grating (1200 grooves per mm) blazed at 5000 Å. Handling of reagents before initiation of the luciferase/luciferin reaction was done under extremely low lighting levels.

Cells and Virus. Equine dermal cells (ATCC CCL57) were grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum and antibiotics. The MA-1 isolate (23) of equine infectious anemia virus (EIAV) was used in all assays.

Titration of Infectious Virus. Cell-free stocks of EIAV containing $\approx 10^5$ focus-forming units (FFU) of EIAV per ml were diluted 1:10 in Hanks' buffered saline solution in 24-well tissue culture plates; hypericin, luciferin, and luciferase were added to the final concentrations indicated in the figure legends. Chemiluminescence was initiated by the addition of ATP. Plates were wrapped with aluminum foil and incubated 30 min at room temperature. Controls included samples in which hypericin or luciferase was omitted and samples incubated in ambient room light. Virus infectivity was quantitated by a focal immunoassay similar to that described (3, 23, 24). Results are expressed in terms of FFU per ml of supernatant. All experimental manipulations were done in subdued light.

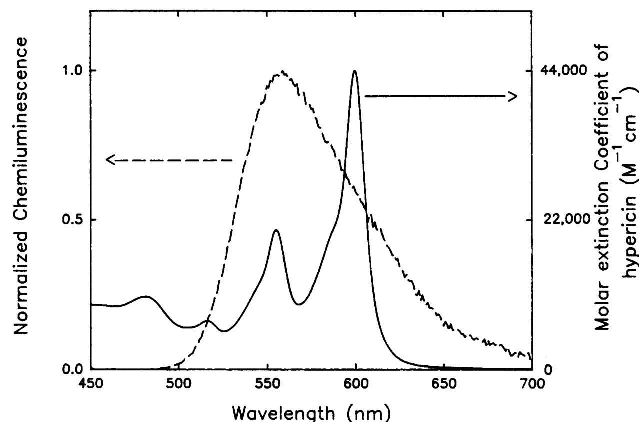

FIG. 3. Spectral overlap between the visible portion of the absorption spectrum of hypericin and the chemiluminescence from the luciferase-catalyzed oxidation of luciferin. The reaction is carried out at 25°C in glycylglycine buffer containing 2.67×10^{-7} M luciferase, 1.18×10^{-6} M luciferin, and 5×10^{-5} M ATP. The efficiency of the nonradiative energy transfer in a Förster energy transfer mechanism is given by R_o, the critical distance. R_o is the distance at which the rate of energy transfer is equal to the inverse of the fluorescent lifetime of the donor, $k_{ET} = (1/\tau_F)\,(R_o/R)^6$, for randomly oriented donors and acceptors. R_o can be calculated from the fluorescence spectrum of the donor and the absorption spectrum of the acceptor (20–22)

$$R_o^6 = \frac{9000(\ln 10)\phi_D}{128\pi^5 n^4 N}\,\frac{2}{3}\int_0^\infty F_D(\nu)\varepsilon_A(\nu)\nu^{-4}d\nu,$$

where n is the index of refraction of the medium, N is Avogadro's number, ϕ_D is the fluorescence quantum yield of the donor, $F_D(\nu)$ is the spectrum of the donor emission normalized to one on a wavenumber scale, and $\varepsilon_A(\nu)$ is the decadic molar extinction coefficient (in liter·mol^{-1}·cm^{-1}) on a wavenumber scale.

RESULTS

First it is necessary to compare the chemiluminescent emission of the luciferase-catalyzed oxidation of luciferin and the absorption spectrum of hypericin in the red region of the visible spectrum (Fig. 3). The high degree of overlap between

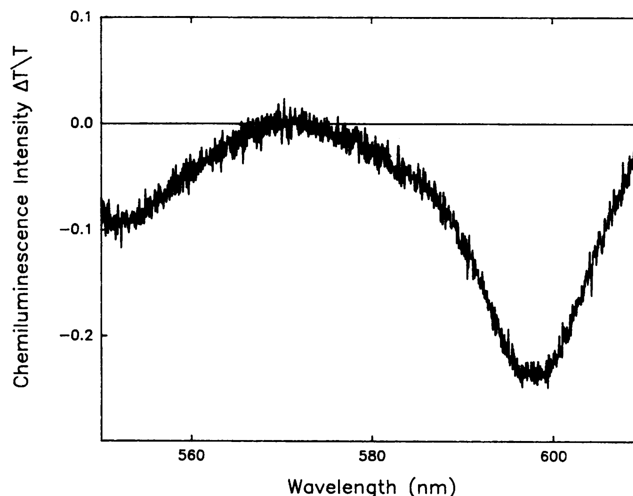

FIG. 4. Attenuation of chemiluminescent emission by hypericin. The figure represents the difference between the light intensity as a function of the wavelength of the chemiluminescent reaction of luciferase and luciferin in the absence and presence of hypericin, ΔT, divided by the light intensity of this reaction in the absence of hypericin, T. Data are thus plotted as a transmission change due to the presence of the energy acceptor hypericin. In the absence of hypericin, the difference of two consecutive runs yields a trace that is centered about zero. The decreases in transmission, of which the most notable is at ≈600 nm, parallel the absorption maxima of hypericin (see Fig. 3).

these spectra suggests that the chemiluminescent emission generated from the luciferin/luciferase reaction is capable of photoactivating hypericin. Calculations based on Förster theory suggest that the so-called "critical distance" for energy transfer between these two species is ≈100 Å (see legend to Fig. 3). The critical distance, R_o, is the distance at which the rate of energy transfer is equal to the rate at which the excited state of the donor decays. In other words, R_o is the distance at which the rate of energy transfer is equal to the rate of fluorescence decay

$$k_{ET} = k_F \left(\frac{R_o}{R}\right)^6,$$

where

$$k_F = \frac{1}{\tau_F},$$

τ_F is the fluorescence lifetime, and R is the separation between randomly oriented donors and acceptors (20–22). The large value of 100 Å obtained for R_o is partly a result of the high degree of spectral overlap between the chemiluminescent emission, but it is also a result of the extremely efficient yield of chemiluminescence. Approximately one photon is produced for every molecule of luciferin (14–18); that is, the quantum yield of the donor, ϕ_D, is unity. Comparable values of R_o are observed for the pigments that constitute donor–acceptor pairs for energy transfer in photosynthesis (22). Therefore, energy transfer between luciferin/luciferase and hypericin is possible even when the donor and the acceptor are not constrained to be at a fixed distance or orientation with respect to each other. These results immediately suggest the possibility of exciting hypericin by means of a chemiluminescent reaction to exploit its photoinduced virucidal activity without an external light source.

Figure 4 indicates that, in the presence of hypericin, the chemiluminescent emission of the luciferase/luciferin reaction is attenuated in the region corresponding to the absorption spectrum of hypericin. This verifies that there is at least a radiative absorption of the chemiluminescent emission by hypericin. In order to show that a nonradiative energy transfer to hypericin occurs, further work will be required to demonstrate that the intensity of the chemiluminescent emission is uniformly reduced in regions where there is no absorption from hypericin.

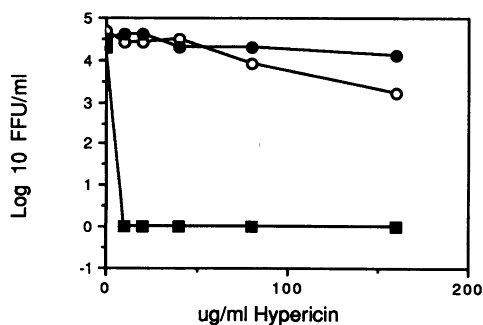

FIG. 5. Effect of chemiluminescence on the antiviral activity of hypericin. EIAV was incubated in the dark at room temperature in the presence of 0.8 μM luciferase, 10 μM luciferin, 2 mM ATP, and increasing amounts of hypericin (○). Control samples include those containing virus and hypericin only (●) and parallel samples exposed to ambient room light (■). Infectious virus was titrated by using a focal immunoassay, and the results are reported as FFU per ml of reaction mixture.

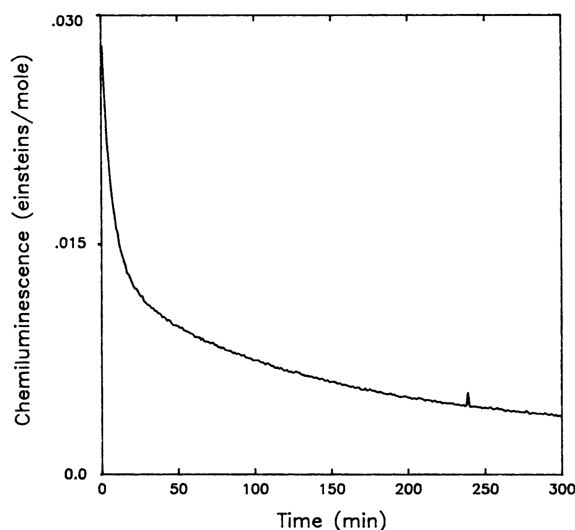

FIG. 6. Time course of the chemiluminescent reaction of luciferin and luciferase.

To test the idea that the chemiluminescent reaction can induce virucidal activity in hypericin, cell-free EIAV was treated with various concentrations of hypericin in a 1-ml solution containing luciferin and luciferase (Fig. 5). Reaction mixtures were incubated 30 min at room temperature in the dark and inoculated onto equine dermal cells in the presence of Polybrene (8 μg/ml). At high concentrations of hypericin, there is an ≈10-fold reduction of viral infectivity under conditions where the sole source of excitation was the chemiluminescent luciferin/luciferase system. The chemiluminescent light-generating system was not, however, as effective in activating hypericin as illumination from a continuous source. A major difference in the light output from the chemiluminescent reaction and, for example, ambient fluorescent light is that the light intensity from the chemiluminescent reaction decreases with time. Fig. 6 depicts the rapid decay in chemiluminescence after luciferase-catalyzed oxidation of luciferin. Further experiments were done to determine whether increased antiviral activity could be achieved by an increase in the amount of light initially available for hypericin activation. A linear decrease in viral infectivity was observed when the concentration of luciferase was varied in the presence of a constant concentration of hypericin (Fig. 7). This suggests that optimal activation of

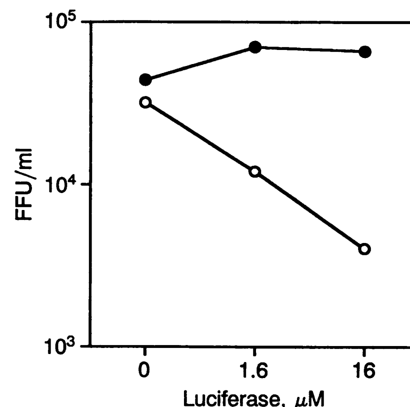

FIG. 7. Effect of luciferase concentration on antiviral activity of hypericin after chemiluminescence. EIAV was incubated with 0 (●) or 10 (○) μg of hypericin per ml in the presence of 5 mM luciferin, 2 mM ATP, and increasing amounts of luciferase. Reaction mixtures were incubated in the dark for 30 min at room temperature, and results are reported as FFU per ml of reaction mixture.

hypericin depends on the local concentration of energy donors.

DISCUSSION

Hypericin is a naturally occurring photosensitizer that displays potent antiviral activity against a variety of clinically important enveloped viruses, including HIV-1. One drawback to the use of hypericin and other photosensitizers as effective chemotherapeutic agents for treatment of viral infections *in vivo* is the requirement for light for optimal virucidal activity. A possible approach to circumvent this problem is the development of methods for chemiluminescent activation of hypericin *in vivo*. In the present report, we have demonstrated that the chemiluminescent reaction of luciferin and luciferase produces a sufficient amount of light to bleach the absorption spectrum of the virucidal agent hypericin even when there is no covalent attachment between these two reactants. Most importantly, the amount of light transferred to hypericin under these conditions is sufficient to produce significant antiviral activity. It is important here to stress that the mechanism of activation of hypericin is not necessarily the same in the two cases. Activation of hypericin by a light source, such as a projector bulb, involves the emission of a photon from the source and its subsequent absorption by hypericin. In the chemiluminescent reaction between luciferin and luciferase, an excited-state singlet, oxyluciferin, is produced (19). In addition to being able to transfer its energy radiatively to hypericin (Fig. 4), oxyluciferin is in principle capable of being deactivated *nonradiatively* by Förster energy transfer to hypericin (Fig. 3).

The chemiluminescent system is not as effective as a continuous source of illumination (Fig. 5) in activating the antiviral activity of hypericin. This is most likely a result of suboptimal distance and orientation between the donor and acceptor. Thus, the antiviral activity of hypericin increases proportionally with increasing concentrations of luciferase, providing further evidence that the limitation of the chemiluminescent reaction is the availability of localized concentrations of acceptable energy donors, which specifically interact with hypericin. Therefore, the proximity of the reactants may be more crucial for the virucidal activity than the concentration of any one reactant. Any *in vivo* application of chemiluminescent activation of hypericin would require a delivery system that ensures a high local concentration of hypericin and luciferin/luciferase.

It is possible that in some cases the reduced antiviral activity of hypericin when using chemiluminescence as compared to an external light source may result from the consumption of oxygen by the luciferase/luciferin reaction (see legend to Fig. 2). In the presence of oxygen, hypericin produces singlet oxygen very efficiently [with a quantum yield of 0.73 (9)], and some studies have suggested that its antiviral activity is due to the production of singlet oxygen (4–6). If so, localized depletion of oxygen by luciferin/luciferase may be expected to reduce the antiviral activity of hypericin. We (13, 25–27) have, however, questioned the relative importance of singlet oxygen in the toxicity of hypericin toward HIV and related viruses. For example, hypericin is closely related (28), both structurally and spectrally, to the photoreceptor of the protozoan ciliates *Stentor coerulus* and *Blepharisma japonicum* (28, 29). This photoreceptor confers upon the organism its biologically necessary photophobic and phototactic responses. Under conditions of ambient light the stentorin chromophore and hypericin are nontoxic to the organism. On the other hand, the singlet oxygen produced from these chromophores is toxic to *S. coerulus* under high light flux ($\approx$5000 W/m^2) (30). It is an open question, therefore, whether the virucidal activity of hypericin after limited exposure to room light (3–6) is due to photosensitized generation of singlet oxygen by hypericin or the presence of additional nonradiative decay processes of the excited states of hypericin. Recent studies in our laboratory indicate that oxygen is not required for antiviral activity of hypericin, although in some cases it may play a role (13). An alternative origin for the photoinduced antiviral activity of hypericin may lie in its ability to produce a photogenerated pH drop, as is observed with the stentorin chromophore (31, 32). We have identified rapid intramolecular proton transfer in hypericin (25–27), which is likely to precede the solvent acidification. Furthermore, several investigations have documented the importance of pH in the replication cycle of certain enveloped viruses by regulating uncoating (33–35).

The finding that the antiviral activity of hypericin can be activated by chemiluminescent reactions may have important implications for the development of methods for treatment of viral infections such as HIV-1. *In vivo* generation of luciferase could be accomplished by gene therapy approaches that use luciferase as a susceptibility gene. Moreover, expression of the luciferase gene could be regulated if placed under the control of a promoter containing HIV-1 TAR sequences, limiting photoactivation of hypericin to virus-infected cells. This would result in a "molecular flashlight" in which light is turned on or off depending on the presence of a transacting viral protein. Recent studies demonstrating the tumoricidal effects (36) of hypericin suggest that similar technology could be applied to gene therapy approaches for the treatment of cancer. The present work is concerned with a theoretical approach to antiviral therapies; more practical issues must await further experimentation. Further efforts to optimize the energy transfer between luciferin and hypericin are needed to improve the feasibility of the molecular flashlight as a viable antiviral and anticancer therapy.

We thank Yvonne Wannemuehler for technical assistance. J.W.P. is an Office of Naval Research Young Investigator. M.J.F. is supported by an Augmentation Award for Science and Engineering Training grant from the Office of Naval Research. Portions of this work were funded by Carver grants awarded to both G.A.K. and S.C. and to J.W.P.

1. Haynes, B. F. (1993) *Science* **260**, 1279–1286.
2. Johnston, M. I. & Hoth, D. F. (1993) *Science* **260**, 1286–1293.
3. Carpenter, S. & Kraus, G. A. (1991) *Photochem. Photobiol.* **53**, 169–174.
4. Meruelo, D., Lavie, G. & Lavie, D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5230–5234.
5. Degar, S., Prince, A. M., Pascual, D., Lavie, G., Levin, B., Mazur, Y., Lavie, D., Ehrlich, L. S., Carter, C. & Meruelo, D. (1992) *AIDS Res. Hum. Retroviruses* **8**, 1929–1936.
6. Lenard, J., Rabson, A. & Vanderoef, R. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 158–162.
7. Hudson, J. B., Lopez-Bazzocchi, I. & Towers, G. H. N. (1991) *Antiviral Res.* **15**, 101–112.
8. Duran, N. & Song, P.-S. (1986) *Photochem. Photobiol.* **48**, 677–680.
9. Racinet, H., Jardon, P. & Gautron, R. (1988) *J. Chim. Phys. Phys. Chim. Biol.* **85**, 971–977.
10. Redepenning, J. & Tao, N. (1993) *Photochem. Photobiol.* **58**, 532–535.
11. Diwu, Z. & Lowen, J. W. (1993) *Free Radical Biol. Med.* **14**, 209–215.
12. Weiner, L. & Mazur, Y. (1992) *J. Chem. Soc. Perkins Trans.* **1**, 1439–1442.
13. Fehr, M. J., Carpenter, S. L. & Petrich, J. W. (1994) *Bioorg. Med. Chem. Lett.* **4**, 1339–1344.
14. Hopkins, T. A., Seliger, H. H., White, E. H. & Cass, M. W. (1967) *J. Am. Chem. Soc.* **89**, 7148–7149.
15. McElroy, W. D. & DeLuca, M. (1981) in *Bioluminescence and Chemiluminescence*, eds. DeLuca, M. & McElroy, W. D. (Academic, New York), pp. 179–186.

16. McCapra, F. M. & Perring, K. D. (1985) in *Chemi- and Bioluminescence*, ed. Burr, J. G. (Dekker, New York), pp. 359–386.
17. White, E. H., Wörther, H., Seliger, H. H. & McElroy, W. D. (1966) *J. Am. Chem. Soc.* **88**, 2015–2019.
18. Seliger, H. H. & McElroy, W. D. (1960) *Arch. Biochem. Biophys.* **88**, 136–141.
19. Koo, J.-A., Schmidt, S. P. & Schuster, G. B. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 30–33.
20. Förster, T. (1965) in *Modern Quantum Chemistry Part III: Action of Light and Organic Crystals*, ed. Sinanoglu, O. (Academic, New York), p. 93.
21. Michel, J. & Bonačić-Koutecký, V. (1990) *Electronic Aspects of Organic Photochemistry* (Wiley, New York), pp. 81–86.
22. van Grondelle, R. & Amesz, J. (1986) in *Light Emission by Plants and Bacteria*, eds. Govindjee, Amesz, J. & Fork, D. C. (Academic, Orlando, FL), pp. 191–223.
23. Carpenter, S. L., Evans, L. H., Sevoian, M. & Chesebro, B. (1987) *J. Virol.* **61**, 3783–3789.
24. Kraus, G. A., Pratt, D., Tossberg, J. & Carpenter, S. L. (1990) *Biochem. Biophys. Res. Commun.* **172**, 149–153.
25. Gai, F., Fehr, M. J. & Petrich, J. W. (1993) *J. Am. Chem. Soc.* **115**, 3384–3385.
26. Gai, F., Fehr, M. J. & Petrich, J. W. (1994) *J. Phys. Chem.* **98**, 5784–5795.
27. Fehr, M. J. & Petrich, J. W. (1994) *J. Phys. Chem.* **98**, 8352–8358.
28. Tao, N., Orlando, M., Hyon, J.-S., Gross, M. & Song, P.-S. (1993) *J. Am. Chem. Soc.* **115**, 2526–2528.
29. Cubbedu, R., Ghetti, F., Lenci, F., Ramponi, R. & Taroni, P. (1990) *Photochem. Photobiol.* **52**, 567–573.
30. Yang, K.-C., Prusti, R. K., Walker, E. B., Song, P.-S., Watanabe, M. & Furuya, M. (1986) *Photochem. Photobiol.* **43**, 305–310.
31. Song, P.-S., Walker, E. B., Auerbach, R. A. & Robinson, G. W. (1981) *Biophys. J.* **35**, 551–555.
32. Walker, E. B., Lee, T. Y. & Song, P.-S. (1979) *Biochim. Biophys. Acta* **587**, 129–144.
33. Pinto, L. H., Holsinger, L. J. & Lamb, R. A. (1992) *Cell* **69**, 517–528.
34. Marsh, M. & Helenius, A. (1989) *Adv. Virus Res.* **36**, 107–151.
35. Zhirnov, O. P. (1990) *Virology* **176**, 274–279.
36. Thomas, C. & Pardini, R. S. (1992) *Photochem. Photobiol.* **55**, 831–837.