Table V. Exceptions to Eq 2 As Compared to the Total Number of Possible Relations in Table IV

relation	β-trypsin	plasmin	thrombin
$S_I^4 \geq S_I^3$	12/16	11/12	12/16
$S_I^4 \geq S_{II}^3$	6/20	4/20	2/20
$S_I^4 \geq S_{II}^4$	0/20	0/20	0/16
$S_I^3 \geq S_{II}^3$	4/20	1/15	0/20
$S_I^3 \geq S_{II}^4$	0/20	0/15	0/16
$S_{II}^3 \geq S_{II}^4$	1/25	3/25	0/20
$S_I \geq S_{II}$	10/80	5/70	2/72

hibitory potency of various substituted derivatives can be ordered as follows:

$$S_I^4 \geq S_I^3 \geq S_{II}^3 \geq S_{II}^4 \quad (2)$$

Upper indices denote substituent positions. Table V illustrates the validity of eq 2. There is only one relation, $S_I^4 \geq S_I^3$, where it is not fulfilled. The less rigorous $S_I \geq S_{II}$ classification (with no respect to substituent position) is valid in 90% of the possible comparisons.

Table V includes data for plasmin and thrombin, too. The three-dimensional structure of these enzymes, also members of the serine proteinase family, is not known at present. However, using computer-generated models, Furie and co-workers have shown that the internal structure and active site of thrombin are similar to β-trypsin.[37] This should mean that the protein electrostatic potential near the active site is also similar in these enzymes;[22] i.e., eq 2 holds also for thrombin. Tables IV and V show that this is the case. Furthermore, eq 2 is fulfilled for plasmin, another important serine proteinase. This indicates that the plasmin active site and "electrostatic lock" should be similar to those of β-trypsin and thrombin.

Conclusions

The present paper describes the quantum chemical calculation of the enzyme–ligand interaction energy for a series of substituted benzamidine inhibitors of β-trypsin. To our knowledge this is the first study which considers the protein in full; others either use empirical methods or model the whole system by a limited number of amino acid residues.[1-8] Our main conclusions are the following.

(1) The experimental Gibbs free energy of association changes parallel with the calculated interaction energy. A similar, linear, dependence is observed between ΔG_{exptl} and the hydration energy.

(2) The "electrostatic lock", representing the active site, is characterized. The electrostatic potential of the enzyme is lower at the amidine and C4 substituents while it is larger along the phenyl ring. The charge pattern of the inhibitor fits into this lock as a key.

(3) By use of the electrostatic lock as a guide simple structure–activity relationships are derived. For X1X2Z type substituents, where X1 is directly attached to the ring and X2 to X1, it is the direction of the X1X2 polarity which determines activity. If X1 is the more negative (OH, OCH_3, etc.), the inhibitory activity is larger than in the case where X1 is the more positive (NO_2, $COCH_3$, etc.).

Registry No. 4-Aminobenzamidinium, 57867-44-4; 4-hydroxybenzamidinium, 57867-43-3; benzamidinium, 53356-58-4; 4-methylbenzamidinium, 57867-49-9; 4-fluorobenzamidinium, 57867-48-8; 4-chlorobenzamidinium, 57867-47-7; 4-nitrobenzamidinium, 57867-51-3; β-trypsin, 9002-07-7.

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Flavin and 5-Deazaflavin Photosensitized Cleavage of Thymine Dimer: A Model of in Vivo Light-Requiring DNA Repair

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Abstract: This paper presents a model photochemical system for the cleavage of the cyclobutane ring of *cis,syn*-thymine dimer and the production of thymine. Many characteristics of this system mimic the in vivo thymine dimer repair phenomenon attributed to the action of photoreactivation enzymes. Lumiflavin, 5-deazariboflavin, and 8-methoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin will each sensitize dimer cleavage. This cleavage depends on the concentration of both thymine dimer and sensitizer, on the extent of irradiation, and on the wavelength of irradiation. Maximum dimer cleavage occurs when the wavelength of irradiation corresponds to the longest wavelength λ_{max} of the sensitizers. This model system also has a distinct pH dependence; dimer cleavage requires a pH of greater than 10. Initial characterization of the mechanism for dimer cleavage catalyzed by either flavin or 5-deazaflavin is also presented and compared to previously described model systems. A derivative of 8-hydroxy-5-deazaflavin, the chromophore of the *Streptomyces griseus* photoreactivation enzyme, is not able to sensitize thymine dimer cleavage under any conditions presented here. Other electron-rich flavins presented in this report are also unable to catalyze dimer cleavage under model conditions.

Intrastrand *cis,syn*-thymidine dimers are the predominant lesion in UV-irraiated DNA.[2,3] Two distinct mechanisms are used by organisms to repair this lesion. The well-characterized "dark" repair system depends on the action of a dimer-specific endonuclease followed by DNA polymerase.[4] The second system requires the presence of visible light to essentially reverse the adduct forming [2 + 2] cycloaddition of adjacent thymidines. Although this photoreactivation actvity was discovered over 30 years ago,[5] specific proteins (photoreactivation enzyme (PRE),

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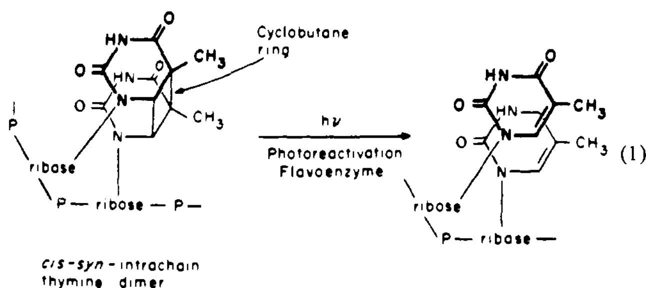
DNA photolyase, EC 4.1.99.3) that catalyze the repair process (eq 1) have only recently been purified from *E. coli*, yeast, and



Streptomyces griseus. The chromophores associated with two of the isolated PREs have been identified; PRE from yeast[6] contains a derivative of riboflavin, and the PRE from *Streptomyces griseus* contains a derivative of 8-hydroxy-5-deazaflavin.[7]

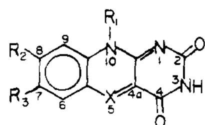
The mechanism by which PRE can convert incident light energy to the chemical energy necessary for cleavage of a cyclobutane derivative and repair of DNA is still not understood. However, a variety of photochemical systems have been developed to study PRE's possible catalytic mechanism. This paper describes the first model system that successfully utilizes both natural and synthetic flavin derivatives to photosensitize the cleavage of thymine dimer.

Previous model systems have not implicated direct energy transfer from an excited chromophore to thymine dimer during the cleavage process. Rather, transient radical intermediates are most likely produced in all of the known photocleavage systems. Thymine dimerization can be reversed by three distinct photosystems, any one of which may operate by the true biomimetic mechanism.

A redox photosensitized system[8] seems to cleave tetramethylthymine dimer through the interaction of a radical cation of phenanthrene with the ground state of the dimer. In other model photosystems, direct electron transfer between an excited-state sensitizer and thymine dimer has been suggested as the mechanism of thymine production.

Hélène and co-workers have demonstrated that a peptide, lys-trp-lys, and a DNA-binding protein will catalyze the cleavage of thymidine dimer through the reversible donation of an electron from an excited indole of tryptophan to the dimer.[9] In contrast, a number of excited-state compounds with high electron affinities seem to catalyze dimer cleavage by first abstracting an electron from the thymine dimer. Irradiation of $K_3Fe(CN)_6$,[10] UO_2SO_4,[10] or a variety of quinones[11] in the presence of dimerized thymine produces the monomer, thymine.

Model systems containing derivatives of free flavin have also been surveyed for thymine dimer cleavage activity. Eker and



5-deazariboflavin, X = C; R_1 = ribityl; $R_2 = R_3 = CH_3$
8-hydroxy-7,8-didemethyl-5-deazariboflavin, X = C; R_1 = ribityl; R_2 = OH; R_3 = H
8-methyoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin, X = C; $R_1 = CH_2CH_3$; $R_2 = OCH_3$; R_3 = H
lumiflavin, X = N; $R_1 = R_2 = R_3$ = CH
riboflavin, X = N; R_1 = ribityl; $R_2 = R_3 = CH_3$
8-hydoxyriboflavin, X = N; R_1 = ribityl; R_2 = OH; $R_3 = CH_3$

co-workers[7] demonstrated that an N^{10} alkylated derivative of 7,8-didemethyl-8-hydroxy-5-deazaflavin similar to the cofactor of *S. griseus* PRE could photosensitize small amounts of thymine dimer to thymine. Yet, Lamola[12] found that derivatives of yeast PRE's cofactor, lumichrome of lumiflavin, could not sensitize dimer cleavage at pH 7. Since flavin and 5-deazaflavin derivatives are clearly involved in the in vivo repair of thymidine dimer lesions, a systematic study on flavin and 5-deazaflavin sensitized dimer cleavage was initiated in our laboratory. This paper describes thymine dimer photolysis catalyzed by flavin and 5-deazaflavins and serves as a prelude to future studies on the mechanism of flavin-containing PREs.

Results

Survey of *cis,syn*-Thymine Dimer Cleavage Activity Catalyzed by Flavins and 5-Deazaflavins. An anaerobic, buffered solution of *cis,syn*-[^{14}C]thymine dimer and photosensitizer was irradiated at the λ_{max} of the photosensitizer. The extent of the cleavage reaction was assessed by measuring the production of monomer [^{14}C]thymine (see Experimental Section). All sensitizers used in this study were surveyed for dimer photolysis over a range of pH and sensitizer concentrations. Those compounds that catalyzed detectable dimer cleavage are characterized below (lumiflavin, 5-deazariboflavin, and 8-methoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin). The N^{10}-alkyl derivatives of these chromophores were used whenever possible in order to simplify the model system. The N^{10}-ribityl derivatives of 5-deazaflavin and other flavins were used due to their ready availability.[13] The presence of the N^{10}-ribityl group per se does not appear to alter the photochemistry of the sensitizer; addition of free ribose to irradiation samples containing 5-deazariboflavin or lumiflavin had no effect on the production of thymine.

No dimer cleavage was detected after photosensitization with the N^{10}-ribityl or N^{10}-ethyl derivative of the *S. griseus* PRE chromophore, a N^{10}-modified 8-hydroxy-7,8-didemethyl-5-deazaflavin. The irradiation conditions varied from pH 4 to 12, and sensitizer concentrations were tested at levels ranging from 10-fold lower to 5-fold higher than the concentration of dimer. This 8-hydroxy-5-deazaflavin, like lumiflavin and 5-deazariboflavin, does not sensitize conversion of the product of cleavage, thymine, back to thymine dimer in contrast to the reported action of some ketones.[14] Therefore, the lack of detectable photolysis in this case is not the result of an efficiently catalyzed back reaction. Infrequently, cleavage activity was detected in certain incubations, but only if a large excess of sensitizer was present and extensive sensitizer decomposition had occurred. Hence, no dimer cleavage could be directly attributed to the 8-hydroxy-5-deazaflavin in this model system.

As demonstrated below, both lumiflavin (λ_{max} = 450 nm) and 5-deazariboflavin (λ_{max} = 400 nm) are well-defined sensitizers for this photolysis. Therefore, the addition of the hydroxyl group at the 8-position of 5-deazariboflavin is responsible for the inactivity of 8-hydroxy-5-deazariboflavin. Some other flavin derivatives that contain an electron-rich substituent at the 8-position, 8-hydroxyriboflavin (λ_{max} = 470 nm), 8-(dimethylamino)riboflavin (λ_{max} = 490 nm), and 8-(methylamino)riboflavin (λ_{max} = 490 nm), were also unable to sensitive dimer cleavage under anaerobic conditions. Common to all of the inactive flavins thus far studied is a substitution at the 8-position that shifts the visible region λ_{max} of the compounds to wavelengths higher than the corresponding λ_{max} of the parent flavin or 5-deazaflavin.

5-Deazariboflavin and Lumiflavin Photosensitization: Dependence on Extent of Irradiation. Samples containing buffer, thymine dimer, and either 5-deazariboflavin or lumiflavin were irradiated

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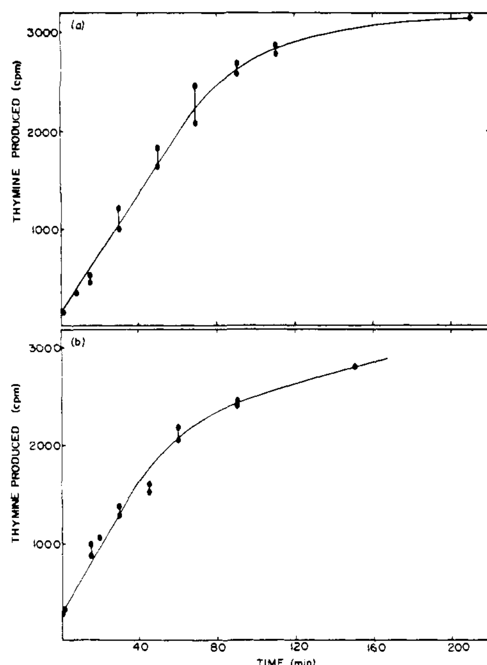

Figure 1. Extent of thymine dimer cleavage vs. the length of irradiation in the presence of (a) 5-deazariboflavin and (b) lumiflavin. Each data point represents the [^{14}C]thymine produced in an aliquot (60 μL) of an irradiation mixture containing on the order of 100 μM sensitizer, ca. 100 μM [^{14}C]thymine dimer (specific activity = 0.42 nCi/nmol), and 100 mM potassium phosphate (pH 12 for 5-deazariboflavin and pH 13.5 for lumiflavin). Samples containing 5-deazariboflavin were irradiated at 400 nm (16-nm band-pass), and samples with lumiflavin were irradiated at 450 nm. Product analysis is described in the Experimental Section.

for varying lengths of time at 400 nm (5-deazariboflavin) or 450 nm (lumiflavin), respectively. The data (Figure 1a,b) clearly demonstrate that the extent of the cleavage depends on the length of irradiation. This dependence is equivalent to a dependence on the total number of incident photons because the dimer cleavage reaction is strictly a light reaction. The reaction cannot be initiated in the dark nor will it sustain itself after an initial period of irradiation.

Although the rate of product formation decreased over time, the rate remained relatively constant for the first 60 min of irradiation using either 5-deazariboflavin or lumiflavin as the sensitizer. In this period, 35–45% of the thymine dimer is converted to thymine. A convenient irradiation time of 20–30 min has been used for studying characteristics of the photolyses discussed in the following sections; the amount of product formed over this period identifies the rate or the relative quantum efficiency of the systems. A 30-min irradiation of 5-deazariboflavin or lumiflavin and dimer under conditions similar to those of Figure 1 produced thymine with a quantum yield between 10^{-3} and 10^{-4}. The upper limit of the quantum yields for these model reactions have not yet been measured.

The decrease in the efficiency of dimer cleavage on prolonged irradiation is likely due to thymine dimer consumption and possible sensitizer degradation. Accumulation of thymine does not inhibit the photoreaction, nor are there any compounds formed during irradiation that quench the dimer sensitization. The extent of dimer cleavage on prolonged irradiation will rise at least 50% over the level extrapolated from Figure 1, if, after an initial 2-h irradiation, additional amounts of thymine dimer, sensitizer, or a combination of both are added before the irradiation is continued.

Over the time period indicated in Figure 1, ca. 50% (3 nmol) of the thymine dimer is converted to thymine in the presence of either 5-deazariboflavin or lumiflavin. With 5-deazariboflavin as the sensitizer, complete dimer cleavage was never achieved under conditions similar to those described in Figure 1, even after an irradiation of 17.5 h. Sensitizer degradation is the most likely cause of the limited reaction. If anthraquinone-2-sulfate is used as sensitizer, 92% of the initial thymine dimer is monomerized after a 17-h irradiation at 330 nm (16-nm band-pass). This is

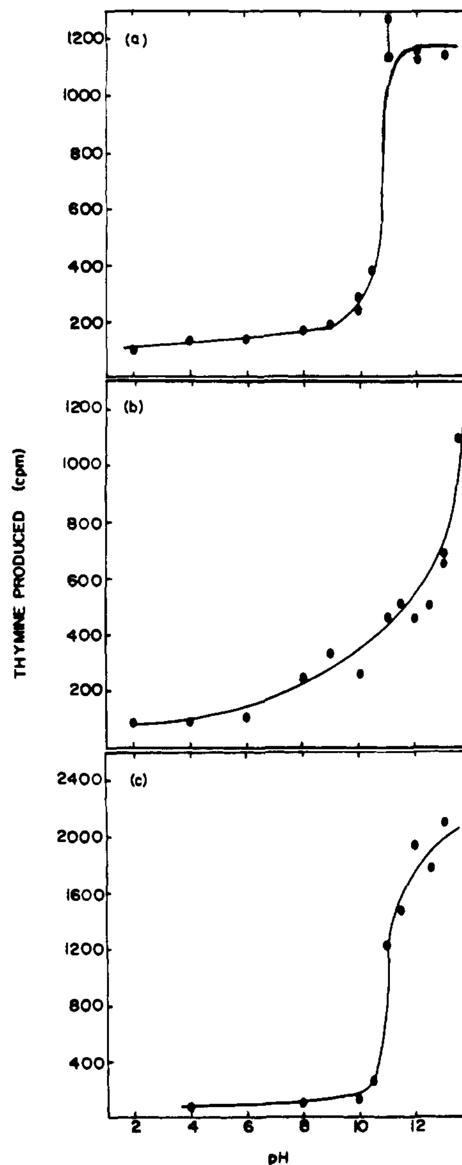

Figure 2. pH dependence of thymine dimer cleavage sensitized by (a) 5-deazariboflavin, (b) lumiflavin, and (c) 8-methoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin. Anearobic photolysis mixtures at the indicated pH (100 mM potassium phosphate) were irradiated at (a) 400, (b) 450, and (c) 383 nm for 20 min. Products were analyzed as described in the Experimental Section. The concentrations of 5-deazariboflavin, lumiflavin, and [^{14}C]thymine dimer in (a) and (b) were the same as those described in Figure 1. The incubations (40 μL) of (c) contained 8.5 μM 8-methoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin and 40 μM [^{14}C]-thymine dimer (specific activity = 2.95 nCi/nmol).

comparable to the published figure of 82%[11] and suggests that the failure of the 5-deazariboflavin reaction to proceed to completion is not an artifact of the incubation conditions.

pH Dependence of Thymine Dimer Cleavage Sensitized by 5-Deazariboflavin, Lumiflavin, and 8-Methoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin. Previous studies[12] had failed to detect the sensitizing capability of lumiflavin because its activity is extremely pH dependent. Figure 2 shows the amount of thymine formed during irradiation of dimer in the presence of either 5-deazariboflavin, lumiflavin, or 8-methoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin at various pH values. For each sensitizer the photolytic reaction only proceeds under highly basic conditions.

This pH dependence is a property of the photosensitized reaction and not of any base-catalyzed dark reaction. Under control conditions at high pH, neither thymine dimer nor the sensitizers decompose. Furthermore, the concentration of the phosphate buffer (100 mM) used in the photolyses and its relative buffering capacity at various pH values do not affect the illustrated pH profiles. 5-Deazariboflavin-sensitized photolyses that contain lower

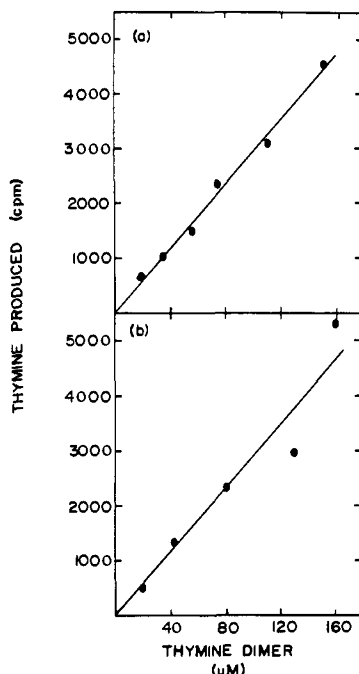

Figure 3. Dependence of sensitized thymine dimer cleavage on the concentration on thymine dimer in the presence of (a) 5-deazariboflavin and (b) lumiflavin. Samples in panel a were individually deoxygenated and irradiated at 400 nm for 20 min; they contained 100 mM potassium phosphate pH 12, 66 μM 5-deazariboflavin, and the indicated amounts of [^{14}C]thymine dimer (specific activity = 4.28 nCi/nmol). Samples in panel b were irradiated at 450 nm for 30 min and contained 100 mM potassium phosphate pH 13.5, 36 μM lumiflavin, and the specified [^{14}C]thymine dimer. The data presented here have been corrected for the increase in background cpm present in the isolated product, thymine, due to the increasing amount of radioactive starting material. For [^{14}C]thymine determination, see the Experimental Section.

concentrations of phosphate (6–50 mM, pH 11) still yield the same amount of dimer cleavage. This reaction, however, will not proceed in the absence of buffer since the pH of the system will not be alkaline. No other buffers have yet been used to replace the phosphate in this sensitized reaction. Finally, the 5-deazariboflavin-catalyzed dimer reversion is not quenched or catalyzed by phosphate pH 11 and, in addition, is not detectably affected by ionic strength.

Effect of Thymine Dimer Concentration on the Photolysis Sensitized by 5-Deazariboflavin and Lumiflavin. The efficiency of thymine dimer cleavage varies linearly under the conditions described in Figure 3. Therefore, this photolysis is first order with respect to dimer when the concentration of dimer and either 5-deazariboflavin or lumiflavin are on the same order of magnitude.

If these sensitizers truly model enzyme catalysis, the dimer dependence should be saturable. Indeed, substrate saturation can be demonstrated when the concentration of 5-deazariboflavin is decreased to 5 μM and the dimer concentration remains similar to that in Figure 3a. At dimer concentrations between 80 and 90 μm, ca. 16-fold greater than 5-deazariboflavin, the cleavage reaction shifts from a first- to zero-order dependence on dimer (Figure 4). Under these conditions, the 5-deazariboflavin-sensitized cleavage also points out another critical feature of the enzyme model; the sensitizer is not consumed in the reaction but acts catalytically. After a 2.3-h irradiation of 160 μM dimer and 5 μM 5-deazariboflavin, 41 μM thymine was produced, indicating that each molecule of 5-deazariboflavin, on average, sensitized the cleavage of four molecules of dimer.

Effect of 5-Deazariboflavin and Lumiflavin Concentration on the Photolysis Reaction. The extent of dimer cleavage exhibited a three-phase dependence on sensitizer concentration. When the concentrations of 5-deazariboflavin or lumiflavin are less than that of the dimer, the photolysis is first order with respect to these sensitizers. In samples that contained ca. 1:1 to greater than 2:1 sensitizer to dimer the photolysis is zero order in sensitizer. This

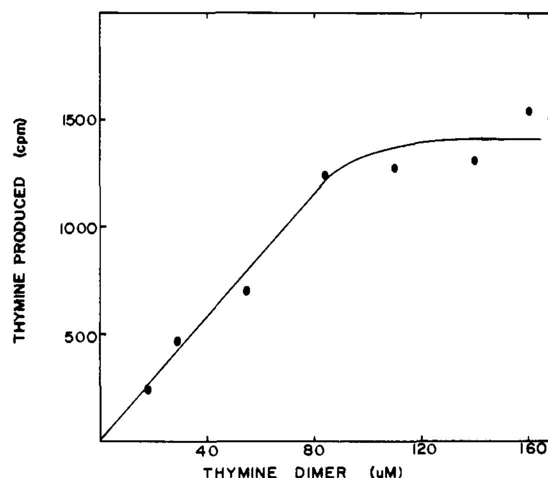

Figure 4. Thymine dimer saturation of the 5-deazariboflavin-sensitized cleavage reaction. The photolyses represented here were identical with those of Figure 3a except the concentration of 5-deazariboflavin was lowered to 5 μM.

held true while varying the concentration of either 5-deazariboflavin or lumiflavin in the range 10–100 μM and using dimer concentrations varying from ca. 20 to 60 μM. If the concentration of 5-deazariboflavin or lumiflavin greatly exceeds the concentration of dimer, the production of thymine is inhibited; the degree of inhibition increases linearly with a rise in sensitizer concentration.

Action Spectra of Thymine Dimer Cleavage Sensitized by 8-Methoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin, 5-Deazariboflan, and Lumiflavin. The sensitizer dependence described above does not, in itself, demonstrate the integral role the sensitizer serves in the cleavage of thymine dimer. As the concentration of a given sensitizer increases, so may the concentration of any trace contaminants or photoproducts. Therefore, the actual photosensitizer of the cleavage reaction is best characterized by the action spectrum of the photolysis.

If the photons absorbed by the 8-methoxy-5-deazaflavin derivative are responsible for the ultimate cleavage of thymine dimer, then thymine production should vary over the wavelength of irradiation according to the absorbance spectrum of the sensitizer. As Figure 5a,b indicates, the excitation of this sensitizer is reponsible for conversion of thymine dimer to thymine. The action spectrum of cleavage mimics the absorbance spectrum of this flavin derivative.

The action spectrum of 5-deazariboflavin-catalyzed dimer cleavage is not as simple as the preceding case. Here, the sensitizer activity vs. wavelength (Figure 5c) is similar to its absorbance spectrum (Figure 5d), but the relative cleavage activity near 340 nm is much less than would be predicted from the deazaflavin's absorbance in this region. Although the cleavage activity between 325 and 355 nm is little more than a shoulder on the peak of main activity, photolyses not presented here more clearly indicate that there is a small peak of activity at 340 nm. However, under no circumstance will an irradiation at 340 nm produce the same amount of dimer cleavage as will a comparable irradition at 400 nm.

The low activity of 5-deazariboflavin irradiated at $\lambda < 360$ nm is not due to a lower flux of incident photons at these wavelengths. In fact, the flux at 366 nm (12×10^{-8} einstein/mol) is even larger than the flux measured at either 436 (4.4×10^{-8} einstein/mol) or 313 nm (4.8×10^{-8} einstein/mol). Yet, the decreased capacity of dimer photosensitization using irradiations of lower wavelengths can still be explained by a real decrease in the number of photons available to 5-deazariboflavin because of a phenomenon unrelated to the photon flux. The decrease in available photons can result from the competitive absorption of a compound or compounds (λ_{max} at 340 nm, see Figure 5d and the following paragraph) produced during the photolysis of 5-deazariboflavin. No photoproduct, however, formally quenches the photolysis reaction (see early section).

Preliminary data suggest that 5-deazariboflavin at high pH predominantly forms a new compound immediately (10 s) after

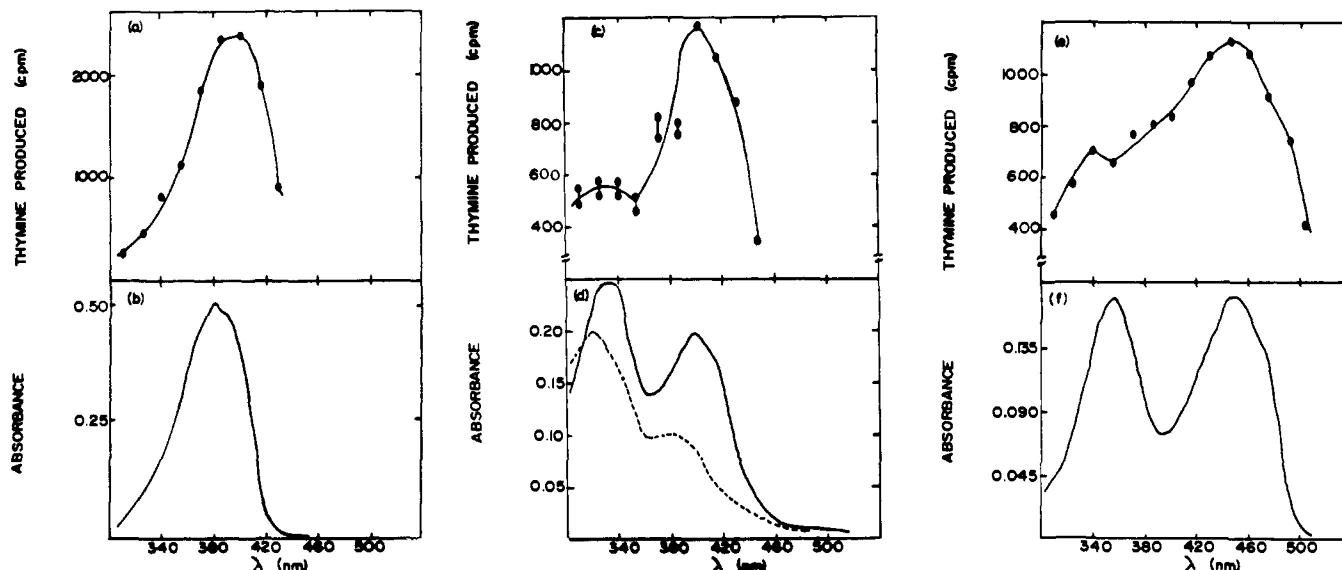

Figure 5. Thymine dimer cleavage action spectra vs. sensitizers' spectra for (a and b) 8-methoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin, (c and d) 5-deazaflavin, and (e and f) lumiflavin. (a) Deoxygenated aliquots (40 μL) of 100 mM potassium phosphate pH 13, 40 μM [^{14}C] thymine dimer (specific activity = 2.95 nCi/nmol), and 8.5 μM 8-methoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin were irradiated for 25 min at the indicated wavelength (16-nm band-pass). [^{14}C]Thymine production was determined here and in the other panels as described in the Experimental Section. (b) Absorbance spectrum of a 34 μM solution of 8-methoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin. (c) Deoxygenated aliquots (40 μL) of 100 mM potassium phosphate pH 12, 42 μM 5-deazariboflavin, and 70 μM [^{14}C]thymine dimer were irradiated for 20 min at the indicated wavelength. (d) Absorbance spectrum of a 5-deazariboflavin-containing irradiation incubation diluted with 500 μL of water before (—) and after (---) an anaerobic irradiation at 400 nm for 1 h. (e) Deoxygenated aliquots (40 μL) of 100 mM potassium phosphate pH 13.5, 31 μM lumiflavin, and 70 μM [^{14}C]thymine dimer were irradiated for 20 min at the indicated wavelength. (f) Absorbance spectrum of a lumiflavin-containing irradiation incubation diluted with 500 μL of water. The action spectra are not normalized for change in the flux of photons vs. wavelength of irradiation.

irradiation. This compound has been detected by using reverse-phase HPLC after 5-deazariboflavin (with or without thymine dimer present) was exposed to 400-nm light. The generation of this product will presumably occur after an irradiation throughout the absorbance spectrum of 5-deazariboflavin. In fact, this photoproduct will form after a 30-min exposure to ambient room light. The fluorescence spectrum of the 5-deazariboflavin photoproduct isolated by HPLC (excitation max = 320 and 355 nm, emission max = 430 nm) is similar to one produced and characterized by Massey and Hemmerich.[15] They have suggested that this compound could be a 8,8′ 5-deazariboflavin dimer, but they have provided no conclusive structural evidence. Since the formation of this 5-deazariboflavin dimer and an alternatively proposed 5,5′ 5-deazariboflavin is readily reversible in the presence of light,[15] a photoequilibrium concentration of 5-deazariboflavin must then remain in the model system to produce an action spectrum with a maximum activity at 400 nm.

The dimer cleavage action spectrum of lumiflavin (Figure 5e) also has a low activity in the region of its lower wavelength λ_{max} (350 nm, Figure 5f). Once again, the greatest extent of dimer cleavage results from irradiation at the visible λ_{max}, ca. 450 nm; the rest of the absorbance spectrum's features are not evident in the action spectrum. A competitively absorbing photoproduct of lumiflavin may also form during lumiflavin irradiation but no such product has yet been detected by light absorbance or by HPLC. If an inactive photoproduct of lumiflavin with a λ_{max} in the region of 350 nm is formed, it must be very labile and must persist only under a constant flux of light.

Chemical Inhibition of Photosensitized Thymine Dimer Cleavage. From the data presented above, an excited state of 8-methoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin, 5-deazariboflavin, and lumiflavin is involved in the mechanism of thymine dimer monomerization. The exact mechanism of this photosensitization is less easily identified but may be revealed by quenching experiments. The fluorescence of 5-deazariboflavin (0.75 μM) and lumiflavin (1.2 μM) is not detectably quenched after the addition of 40 μM thymine dimer, and, therefore, the chromophores in their singlet state probably cannot transfer their energy to thymine dimer. In addition, compounds that do inhibit photosensitized dimer cleavage (discussed below) have no effect on the sensitizers' fluorescence.

Two known quenchers of triplet flavin, oxygen[16] and diazabicyclo[2.2.2]octane (Dabco),[17] inhibit the photolysis catalyzed by either 5-deazariboflavin or lumiflavin. Ambient concentrations of oxygen (250 μM) completely prohibit dimer cleavage. Lower concentrations of oxygen suppress the cleavage reaction but also will subsequently oxidize the nascent thymine, a process now under investigation. Concentrations of Dabco 2-fold higher than that of thymine dimer and 5-deazariboflavin or lumiflavin (ca. 60 μM) inhibits the production of thymine only 15–20%. However, increasing the concentration of dabco to 340 μM suppresses each reaction by at least 75%.

Dabco is thought to quench triplet flavin by reducing it by one electron, producing the flavin semiquinone and the radical cation of Dabco.[17] Under the conditions of these photolyses, the radical cation of Dabco cannot be seen perhaps due to its low absorptivity.[18] Because thymine dimer cleavage is inhibited during the formation of 5-deazariboflavin's or lumiflavin's semiquinone, these species will obviously not catalyze the cleavage reaction.

tert-Butyl alcohol, often used as a free radical scavenger, does not inhibit the sensitized cleavage of thymine dimer at concentrations (250 μM) 4-fold higher than the dimer and sensitizer. n-Butyl alcohol at the same level also did not inhibit the photolysis. Higher concentrations of an organic alcohol, however, did prohibit dimer sensitization. The addition of ca. 15% isopropyl alcohol inhibits the 5-deazariboflavin-catalyzed reaction by 60–80% without affecting the pH.

Discussion

Active Sensitizers. The data presented in this work demonstrate that flavin and 5-deazaflavin are capable of catalyzing thymine dimer photocleavage outside a protein milieu. This model cleavage reaction is a conventional photosensitized reaction, suggesting that catalysis effected by yeast photoreactivation enzyme (containing

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flavin) may also be a classical photosensitization. 5-Deazariboflavin, lumiflavin, and 8-methoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin all display similar properties when sensitizing the cleavage of thymine dimer. Other flavin derivatives such as 8-hydroxyriboflavin, 8-hydroxy-5-deazariboflavin, 8-(dimethylamino)riboflavin, and 8-(methylamino)riboflavin do not sensitize the cleavage reaction using any conditions presented.

The usefulness of this system as a model for PRE is shown by the similarities between the photochemistry of the flavin–thymine dimer system and certain aspects of PRE turnover. Most importantly, the flavin sensitizer acts as a catalyst, absorbing light energy and channeling it to cleave thymine dimer; the sensitizer is not consumed in this reaction.[19] In addition, the rate of the photolysis is saturable by the substrate, thymine dimer. In the early phase of photolysis, product formation is linear over time, once again reminiscent of an enzyme-catalyzed reaction. The time course of the dimer cleavage reaction (Figure 1) indicates that the active sensitizer is present at the start of irradiation and that it does not quickly decompose thereafter.

The photosensitized thymine dimer cleavage can also be saturated with sensitizer but this is most likely caused by quenching reactions such as sensitizer self-quenching.[20] This saturation is evident well before all the incident light is absorbed. Thus, the sensitizer dependence presented here is most accurately described as a dependence on the concentration of both excited- and ground-state sensitizer. Dimer cleavage should increase as the concentration of the excited state of the sensitizer increases. The presence of excess sensitizer in its ground state will, however, compete with thymine dimer to react with the photoexcited sensitizer.

The pH dependence demonstrated in all of the above active photolytic models is remarkable and not evident in any other dimer-cleaving model system. Absolutely no thymine production (above background) is evident until quite high pH. Furthermore, no dark or light-initiated degradation of this system seems responsible for the extreme pH dependence. Two significant characteristics of the flavin–thymine dimer irradiation samples are affected in the pH range 10–12. First, the common substrate of this system, thymine dimer, has a pK_a (10.7) in this region.[21] However, the deprotonation of this dimer cannot be the only pH-dependent property of the active model since the pH profiles presented in Figure 2 do not all reflect only this pK_a. Second, the N^3 proton of flavin[16] and, presumably, the other two active photosensitizers have a pK_a in this region. The significance of this pK_a is not clear since the increased capacity of lumiflavin to sensitize thymine formation does not closely correspond to an increase in the concentration of deprotonated flavin estimated from its pK_a (ca. 10 in both its ground state[16] and its first excited triplet state[22]). Moreover, the amount of triplet flavin produced after light absorption actually decreases with increasing pH,[22] and, thus, the pH profiles of dimer cleavage may actually result from the combination of a variety of separate factors.

The pH dependence of this system might alternatively be a characteristic unique to thymine dimer–flavin molecular complexes that most likely form in solution. Thymine is known to complex riboflavin in solution with a K_{app} of 41×10^2 M.[23] Thymine also seems to self-aggregate in aqueous solutions.[24] In fact, a preassociation of thymine dimer and flavin may be crucial to the ultimate production of thymine. If this is the case, isopropyl alcohol may inhibit this model system because it disrupts the aggregation phenomenon.

Inactive Sensitizers. All the inactive sensitizers contain heteroatoms attached to the C8 position of flavin that can donate electron density into the aromatic ring system. This added electron density is responsible for the red shift in the λ_{max} observed for all of the nonphotosensitizing 8-substituted flavins (with respect to the λ_{max} of the parent flavins). When such donation is blocked, as in the case of the 8-methoxy derivative, the red shift is no longer evident. Furthermore, this O-methylated derivative (λ_{max} = 380 nm) of an inactive sensitizer, 8-hydroxy-5-deazariboflavin (λ_{max} = 420 nm), efficiently sensitizes the cleavage of thymine dimer. The N-dialkylation of 8-aminoriboflavin is not sufficient to block the red shift of its λ_{max} (490 nm) relative to flavin's λ_{max} (450 nm), and, hence, 8-(dimethylamino)riboflavin is predictably unreactive with thymine dimer.

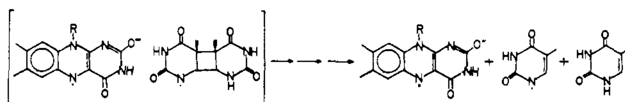
8-(Dimethylamino)riboflavin should also be unreactive as a sensitizer because its singlet and triplet lifetimes are substantially lower than those of unsubstituted flavin.[25] Thus, the electronic properties of 8-hydroxyriboflavin, 8-hydroxy-5-deazariboflavin, 8-(dimethylamino)riboflavin, and 8-(methylamino)riboflavin might alone inhibit sensitization of thymine dimer. Alternatively, these properties may only cause the flavins to be more photolabile than 5-deazariboflavin, lumiflavin, and the 8-methoxy-5-deazaflavin derivative. Preliminary data does, in fact, suggest that the inactive flavins are very sensitive to light (data not shown).

The small yield of thymine (7%) produced after irradiation of an N^{10}-alkylated 8-hydroxy-5-deazaflavin and thymine dimer reported by Eker et al.[7] may actually be dependent on the deazaflavin's decomposition. Under conditions of a normal photolysis reported here, this deazaflavin was consistently inactive as a photosensitizer. Only when excessive sensitizer degradation was apparent did dimer cleavage result. The general photochemistry of 8-hydroxy-5-deazaflavin has not yet been characterized enough to allow an understanding of how a photoreactivation enzyme containing this chromophore can successfully catalyze thymidine dimer cleavage. Selective protonation of the 8-hydroxyl group (pK_a = 6.65)[7] in the enzyme's active site may be important for its activity at physiological pH. The protonated species of 8-hydroxy-5-deazariboflavin has a spectrum (λ_{max} = ca. 380 nm) very similar to that of the 8-methoxy-5-deazaflavin derivative (λ_{max} = ca. 380 nm).

Mechanistic Considerations of This Model for Photoreactivation Enzymes. Photosensitizers, either in an excited singlet or triplet state, may transfer their energy to a substrate in a direct manner or by a transient electron (or hydrogen) shift. The characteristics of the flavin-sensitized thymine dimer cleavage are inconsistent with a direct energy-transfer mechanism from the singlet flavin. The compounds that quench the sensitized cleavage do not affect the fluorescence of the sensitizers.

The triplet flavin is likely to be a key intermediate in the sensitized process since two triplet quenchers, oxygen and Dabco, can prohibit thymine formation. Direct energy transfer, again, seems unlikely between a triplet flavin and thymine dimer. Thyminer dimer (λ_{max} < 200 nm) has no absorbance in the visible region and therefore is unlikely to have a triplet energy level low enough that it could be populated by the low-lying triplet flavin. The probable mechanism of thymine dimer cleavage is then electron (or hydrogen) transfer between a triplet flavin and thymine dimer, a process common to many non-flavin PRE models (such as anthraquinone-2-sulfate and $K_3Fe(CN)_6$).[10,12]

This electron transfer most likely occurs between a dimer and



(19) Under constant illumination, the flavins studied here will react to form new products whose identity and characteristics are now being studied. However, sensitizer degradation proceeds with or without the presence of thymine or thymine dimer. These photoproducts are not then pyrimidine-alkylated flavins.

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closely associated sensitizer. The reaction is inhibited when the solvent properties are altered by addition of isopropyl alcohol, but no inhibition of the photolysis can be detected after addition of a free radical trap. Furthermore, the dimer cleavage does not result from a light-initiated chain reaction; all dimer cleavages stops when an irradiation sample is removed from light.

The direction of electron transfer between the excited flavin and thymine dimer remains to be determined experimentally. Lamola[12] has presented evidence from a variety of experiments that indicates the importance of electron abstraction from thymine dimer by excited-state quinones. Flavins in their triplet state are also known to abstract an electron from some compounds containing nitrogen.[26] Therefore, a reasonable mechanism for this model of PRE activity includes an electron abstraction from the dimer to the triplet flavin to give the illustrated dimer radical and flavin semiquinone as initial intermediates. Only those flavins with relatively low electron density sensitize cleavage; the others with electron-donating groups, including 8-hydroxyriboflavin, 8-hydroxy-5-deazariboflavin, and 8-(alkylamino)riboflavin, cannot. This mechanism depends on an electron abstraction from thymine dimer, a process that should be facilitated at elevated pH and may contribute to the pH profiles of Figure 2. The exact protonation state of the active dimer–sensitive complex is not known. Thus, the complex is shown above with both compounds deprotonated since the pK_a value of each individual compound is lower than the typical pH of the photolyses.

Conclusion

Characterization of the DNA photorepair phenomenon has been hampered by the small amounts of purified photoreactivation enzymes available for study and by the complexity of the required thymine dimer containing DNA substrate. The photochemical system presented here serves as a simple and relatively well-defined model for light-requiring DNA repair. Moreover, the reaction catalyzed by these model systems mimic the rare biological causes of an enzyme-mediated conversion of light energy directly to chemical energy. This model incorporates the essence of the PRE-catalyzed reaction, the capture of visible light by a flavin or 5-deazaflavin and the utilization of its energy to effect the specific cleavage of thymine dimer to thymine. The protein environment of yeast PRE, therefore, is not necessary for successful flavin-catalyzed thymine dimer cleavage, yet that of the *S. griseus* enzyme may be required to stabilize the active form of its 8-hydroxy-7,8-didemethyl-5-deazaflavin chromophore.

Experimental Section

Materials. All materials not explicitly discussed below were of the highest quality commercially available and used without further purification.

***cis,syn*-Thymine dimer** was prepared[27] as needed by the irradiation (254 nm, low-pressure Hg arc) of a frozen thymine (Sigma Chemical Co.) or [methyl-^{14}C]thymine (New England Nuclear) solution, 1 mg/mL for ca. 5 h. The thymine dimer was then purifed from the crude product on an analytical 5-μm C-18 high-performance liquid chromatography (HPLC) column (Alltech) eluted with water (1 mL/min). The isolated thymine dimer was stored as a lyophilized powder. The specific activity of purified [^{14}C]thymine dimer was calculated by measuring the radioactivity of a standard solution of dimer. The concentration of thymine dimer was determined from its absorbance at 210 nm ($\epsilon = 7.6 \times 10^3$).[21] Depending on the individual dimer synthesis, the specific activity varied from 0.45 to 4.28 nCi/nmol.

Anthraquinone-2-sulfate sodium salt and diazaobicyclo[2.2.2]octane (Dabco) were purchased from Aldrich and recrystallized from water before use.

All flavin derivatives except lumiflavin and 8-methoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin used here were a gift from Dr. Ed. Rogers, Wally Ashton, and Don Graham of Merck Sharpe and Dohme and were used without further purification. Each sensitizer was homogenous by analytical reverse-phase C-18 HPLC using a solvent system of 25 or 50 mM potassium phosphate pH 6 with a linear gradient of methanol. Lumiflavin was a gift of Dr. Sandro Ghisla, Konstanz, West Germany, and the 8-methoxy-7,8-didemethyl-5-deazaflavin derivative was a gift from Dr. Lyn Tsai of N.I.H. Both were used without further purification.

Optical Measurements. UV–Vis spectroscopy was performed on a Perkin-Elmer 554 spectrophotometer. Fluorescence measurements were made on a Perkin-Elmer LS-3 spectrofluorimeter.

Irradiation Apparatus. Sensitized thymine dimer irradiations were carried out at room temperature with a 150-W high-pressure Xe arc lamp (Photochemical Research Assoc., 200 series) and a holographic diffraction grating monochromator blazed for an optimum at 450 nm (Photochemical Research Assoc., H-10). The monochromator was set with slit widths of 2 mm allowing for a band-pass of 8 nm on either side of the selected wavelength. Under these conditions, the photolyzed mixtures remained at room temperature even after extensive irradiation. All samples were irradiated at the longest λ_{max} of the sensitizer except when noted; standard irradiation exposures were 20–30 min.

Sample Preparation. The appropriate concentrations of thymine dimer, sensitizer, and potassium phosphate buffer were mixed in a 3-mL Pyrex conical centrifuge tube fitted with a soft rubber stopper. The oxygen was removed from these samples, unless noted, by cycling the contents of the tube between argon and vacuum 6 times. The reactant concentrations presented here were adjusted for the ca. 33% loss in volume resulting from this procedure. Stock concentrations of the potassium buffer were adjusted to the appropriate pH by adding KOH to either phosphoric acid or the various potassium salts of phosphate. All pH values were measured by a Corning Model 12 pH meter. 5-Deazariboflavin-containing incubations (40–60 μL) normally contained 100 mM potassium phosphate pH 12; lumiflavin-containing incubations (40–60 μL) normally contained 100 mM potassium phosphate pH 13.5. All samples were covered with aluminum foil except during the irradiation period noted.

Product Analysis. Irradiation samples were mixed with nonradioactive thymine and thymine dimer and separated by HPLC on an analytical 5-μm C-18 column (Alltech). The sample was eluted (1.5 mL/min) with a 50 mM potassium phosphate pH 6 solution containing 5% methanol and monitored at 214 nm (Waters Assoc. 441 detector) and at 254 nm (Waters Assoc. 440 detector). The material eluting with the thymine dimer standard (retention time of 5.6 min) and thymine (retention time of 7.2 min) was collected, mixed with Liquiscent scintillation fluid (National Diagnostics), and counted on a Beckman LS-100 scintillation counter. Although 5,6-dihydrothymine coelutes with thymine in this HPLC system, thymine is the product of the sensitized irradiations. If no thymine standard is added to the HPLC assay, the product of the photolysis eluting at the position of thymine has a A_{214}/A_{254} (1.3) consistent with thymine but not 5,6-dihydrothymine ($A_{214}/A_{254} > 14$). Any subsequent sensitized photooxidation of the product, thymine, was monitored by collecting and counting the column effluent prior to the elution of thymine dimer where compounds such as the cis and trans glycol of thymine and methylbarbiturate elute.

Quantum Yield Determination. The quantum yield for dimer cleavage was estimated by comparing the number of photons absorbed by 5-deazariboflavin or lumiflavin with the moles of thymine formed over an irradiation period. The quantum yields listed in the text are characteristic of incubations used here; they do not reflect the maximum efficiency possible for this model system. The number of photons absorbed by the system depends on the concentration and absorptivity of the sensitizer. The path length of the irradiation tubes is ca. 3 mm. The flux of incident photons was measured by standard ferrioxalate actinometry.[28,29]

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Registry No. Thymine dimer, 3660-32-0; 8-methoxy-7,8-didemethyl-N^{10}-ethyl-5-deazaflavin, 80547-91-7; lumiflavin, 1088-56-8; 5-deazariboflavin, 19342-73-5; thymine, 65-71-4.

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