Use of the Membrane Binding Technique to Study the Kinetics of Yeast Deoxyribonucleic Acid Photolyase Reactions. Formation of Enzyme–Substrate Complexes in the Dark and Their Photolysis†

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ABSTRACT: The stable complex, which forms in the dark between yeast DNA photolyase and the cyclobutadipyrimidines in far-ultraviolet irradiated T7 [^{3}H]DNA, could be trapped on a nitrocellulose membrane and counted. Using this technique, the rates of association, k_1, and of dissociation, k_2, of the complex were determined, and the calculated values were 2.7×10^7 l. mol^{-1} sec^{-1} (22°) and 1.4×10^{-3} sec^{-1} (22°), respectively. Their ratio yielded a value for K_d ($= k_2/k_1$) of 5.2×10^{-11} mol l.$^{-1}$ (22°). From the values of k_1 and k_2 measured at several temperatures, energies of activation of 4.4 and 6.9 kcal mol^{-1} were computed from Arrhenius plots for the association and dissociation steps, respectively. Under equilibrium conditions complex formation is at a maximum at pH 7.6–7.8 and at an ionic strength of 0.175 (pH 7.5). The action spectrum for photolysis of the complexes was determined by plotting the rate ($\epsilon\Phi$) of their light-catalyzed disruption at several wavelengths, and a maximum occurred at 366 nm, where the value for $\epsilon\Phi$ was 4.9×10^4 l. mol^{-1} cm^{-1}. Treatment of the enzyme with ferricyanide markedly inhibited complex formation, but the enzyme could be partially reactivated with 2-mercaptoethanol. Various other cysteine modifiers also inhibited complex formation, while compounds which attack cystine bridges, *e.g.*, sulfite and dithionite, were ineffective.

Data from several laboratories support the idea that sunlight induces the formation of cyclobutadipyrimidines[1] in DNA, lesions that interfere with DNA replication. Plants, microbes, and some nonplacental mammals (Cook, 1970) contain a unique enzyme, DNA photolyase, requiring light for its activity, capable of undoing this damage and restoring DNA function. The enzyme forms a stable complex in the dark with far-ultraviolet (uv) irradiated DNA (eq 1). Upon

$$\text{dark reaction:} \quad E + \underset{\text{(dipyrimidines)}}{\text{DNA(irr)}} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{[E–DNA(irr)]} \tag{1}$$

illumination of the complex with near-uv or visible light, the uncoupling of the cyclobutane ring, accompanied by restoration of the complexed region of the DNA to its original configuration, frees the enzyme for further DNA repair (eq 2).

$$\text{light reaction:} \quad \text{[E–DNA(irr)]} \xrightarrow{k_3} E + \underset{\text{(less one dipyrimidine)}}{\text{DNA(irr)}} \tag{2}$$

Evidence for the formation of a stable complex stems from kinetic studies, sucrose density sedimentation measurements, and gel filtration experiments (Rupert, 1962a,b). With the data obtained from flash photolysis techniques, Harm and Rupert (1970a) calculated k_1 and k_2, rate constants for the association and dissociation of the complex, respectively. In their work enzyme activity was determined by a transformation assay that measured the capacity of photoenzymatically repaired transforming DNA carrying a streptomycin marker to increase the streptomycin resistance of *Haemophilus influenzae* cells.

Recently, we succeeded in physically separating the photolyase-irradiated T7 [^{3}H]DNA complex on nitrocellulose membranes using the technique elegantly exploited by Riggs and coworkers (1970) to study the binding of the *lac* repressor to its operator and we have modified this technique into a new assay for photolyase (Madden *et al.*, 1973). With this new assay we have now determined the kinetic parameters associated with the binding of photolyase to irradiated DNA, the effects of ionic strength and pH on this binding, and the action spectrum for the photolytic reaction. While previous data pointed to involvement of a sulfhydryl group in photolyase activity, data in this report narrow the involvement of this group to the binding step.

Methods and Materials

Membrane Binding Assay. The procedure used is that of Riggs *et al.* (1970) modified for DNA photolyase assay by Madden *et al.* (1973). The filtering apparatus consists of an 8-in. diameter glass funnel, supported by a rubber stopper in a 1-l. glass vacuum flask and fitted across its mouth with a 0.25 in. thick 70 μ porosity, linear hydrophilic polyethylene sheet (Bel-Arts Product Inc.). The sides of the circular porous disk are taped to the edge of the funnel to improve the vacuum. The apparatus permits filtration through 48 membranes simultaneously.

In the dark a complex forms between the enzyme and T7 [^{3}H]DNA containing dipyrimidine lesions, which is trapped on Schleicher and Scheull B-6 nitrocellulose membranes during filtration. In the dark refers to experiments performed under General Electric gold fluorescent lights which emit nonphotoreactivating light. The dipyrimidines are induced by a germicidal lamp, emitting principally at 254 nm, and their concentration is directly related to the uv dose (Madden *et al.*, 1973). For complete binding of the complex to the membrane an average of five dipyrimidines per DNA genome is required,

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[1] "Dipyrimidine" is used as the trivial substitute for cyclobutadipyrimidine.

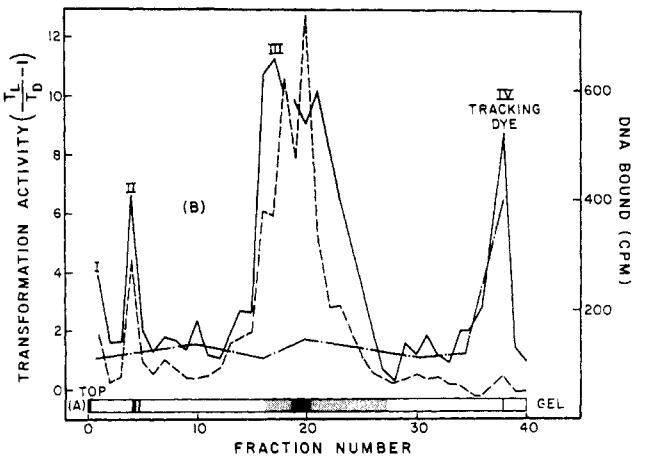

FIGURE 1: Disc gel patterns of protein bands stained with Coomassie Blue (A) and photolyase assays of duplicate unstained gels (B). The enzyme was electrophoresed on 10% polyacrylamide disc gels at pH 8.9 (Davis, 1964). The unstained gels were sliced into 2-mm thick discs and each was extracted with 1 ml of EDB at 0° for 1 hr. Transformation activity (- - - -) is expressed as the ratio ([colonies, light (T_L)/colonies, dark (T_D)] − 1) while binding activity (—) is given by the counts per minute of irradiated T7 [^{3}H]DNA bound. The binding of these fractions to nonirradiated DNA (control) is also indicated (-·-).

the assumption being that each dipyrimidine binds one enzyme molecule. As a control identical samples are run with nonirradiated T7 [^{3}H]DNA substituted for the irradiated substrate.

Enzyme, in a buffer (EDB)[2] consisting of 0.05 M K_2HPO_4, 1 mM EDTA, 1 mM HSEtOH, 0.1% bovine serum albumin (Worthington Biochemical Corp., fraction V), and 5% Me_2SO (pH 7.5), is mixed with an equal volume of DNA solution I, composed of irradiated (10 J/m^2) T7 [^{3}H]DNA, 2.5 μg/ml, and calf thymus DNA, 50 μg/ml, in 0.15 M NaCl. After a 5-min incubation in the dark at 37° when equilibrium is reached, the solution is filtered, and the membrane washed three times with an equal volume of EDB lacking bovine serum albumin. The membranes are dried, dispersed in Permafluor solution B (Packard Instrument Co.), and counted in a liquid scintillation counter.

Transforming Assay for Photolyase. This procedure, involving streptomycin-sensitive *Haemophilus influenzae* cells, has been previously described (Saito and Werbin, 1970).

Preparation of Photolyase. The enzyme is prepared essentially as described by Minato and Werbin (1971) except for the use of a linear gradient of from 0 to 1 M KCl to elute the enzyme during phosphocellulose chromatography. The active fractions, assayed by the binding and the transformation procedures, are then concentrated and dialyzed against 0.05 M Tris (pH 7.5) in an Amicon Hollow Fiber Dialyzer Concentrator, Model DC-2. This active material is chromatographed on a uv-irradiated DNA cellulose column and eluted stepwise with 0.05 M Tris buffer (pH 7.5) containing varying KCl concentrations. Glycerol is added to the active fractions to a concentration of 5%, and they are pooled and concentrated to approximately 0.5 mg/ml in a Model 52 Amicon Stirred Cell under nitrogen. The concentrated photolyase is stable for several months when stored at −70°. Its stability is no doubt related to both the high protein and salt concentrations (Muhammed, 1966). The enzyme has a molecular weight of 53,000 and exhibits maxima at 385 and 485–490 nm

[2] Abbreviations used are: EDB, enzyme dilution buffer; HSEtOH, 2-mercaptoethanol; ES, enzyme–substrate.

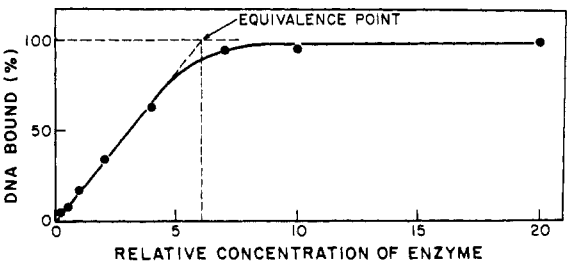

FIGURE 2: Per cent T7 [^{3}H]DNA bound as a function of the relative enzyme concentration. The photolyase was diluted with EDB to various concentrations from 1:2 to 1:2000 and 0.1 ml of each solution was mixed with 0.1 ml of DNA solution I, a 0.15 M NaCl solution containing irradiated (10 J/m^2) T7 [^{3}H]DNA (2.5 μg/ml) and unirradiated calf thymus DNA (50 μg/ml). The mixtures were held at 23° for 5 min and 0.15 ml of each was filtered, washed, and counted. The maximum possible counts per minute of DNA bound was determined by placing a 0.15-ml aliquot of enzyme–DNA solution directly into a vial and counting it.

in its excitation and fluorescence emission spectra, respectively. Preillumination of enzyme that has been stored in the dark at −70° with near-uv or visible light apparently increases the number of active molecules (Harm and Rupert, 1970b). Enzyme used in this study may be considered preilluminated since it is exposed to white fluorescent lighting during thawing and subsequent dilution.

The pattern of protein bands, detected by Coomassie Blue staining (Burgess, 1969) following electrophoresis of the concentrated photolyase preparation on 10% polyacrylamide gels (Davis, 1964), is similar to that seen by Minato and Werbin (1971) except for an additional diffuse band (peak III, Figure 1). A duplicate, unstained gel is cut into 2-mm sections, and each is eluted with 1 ml of EDB for 1 hr at 0°. The results (Figure 1) show that enzyme activity is eluted from all the bands that stained with the dye. The multiplicity of bands probably results from the marked tendency of the enzyme to aggregate into dimers and trimers (Saito and Werbin, 1970; Sutherland *et al.*, 1973).

Estimation of the Number of Active Photolyase Molecules. Five dipyrimidines per genome are introduced into T7 [^{3}H]-DNA by uv irradition, and the amount of DNA bound by increasing concentrations of enzyme is plotted (Figure 2). The intersection of the extrapolation of the linear portion of the curve with the horizontal line representing 100% bound substrate is the point at which the number of enzyme molecules and dipyrimidines are equivalent. This value adjusted by the dilution factor and divided by Avogadro's number provides an estimate of the molarity of the original undiluted enzyme solution. A typical photolyase preparation purified through the affinity chromatography step yielded a value of 1.67×10^{-8} M, which is close to 1.33×10^{-8} M found for the same enzyme preparation by the flash photolysis techniques of Harm and Rupert (1970a).

Determination of the Association Constant for Complex Formation, k_1. When a mixture of complex, free enzyme, and free substrate is filtered on the membrane, only the complex is retained; hence, complex formation can be followed by filtering aliquots of such a solution at intervals after mixing. The complex concentration is calculated from eq 3, where [ES] =

$$[ES] = \frac{[ES]_t \times \text{efficiency factor}}{\text{no. of binding sites}} \qquad (3)$$

concentration of the complex; $[ES]_t$ = (counts per minute (cpm) at time t/cpm_{max}) × total dipyrimidine concentration; efficiency factor = 100/per cent efficiency per binding site

(this refers to the efficiency of binding to the membrane of a DNA molecule containing a single ES complex; in photolyase systems this equals approximately 20% (therefore the efficiency factor $\simeq 5$); and the number of binding sites = 5 dipyrimidines/genome, unless otherwise stated. Therefore, in our system $[ES] = [ES]_t$.[3]

The kinetics for complex formation is given by eq 4,

$$\frac{d[ES]}{dt} = k_1[E]_t[S]_t - k_2[ES]_t \quad (4)$$

where $[E]_t = [E]_0 - [ES]_t$ and $[S]_t = [S]_0 - [ES]_t$. During the initial phase of the reaction $k_2[ES]_t$ is negligible compared to $k_1[E]_t[S]_t$. The calculation of the initial concentration of enzyme molecules $[E]_0$ and of the initial substrate concentration $[S]_0$ in moles of dipyrimidines per liter is described in the previous section. From a plot of irradiated DNA bound *vs.* time, $d[ES]/dt$ and [ES] can be determined leaving k_1 as the only unknown in eq 4.

Determination of k_2, Dissociation Constant of the Complex. The procedure used to determine the dissociation constant is essentially that described by Riggs *et al.* (1970) in which the loss of the complexes is followed in the presence of an excess of unlabeled irradiated DNA added to remove enzyme freed by the dissociation. A solution of irradiated T7 [^{3}H]DNA and a saturating amount of photolyase are equilibrated for 5 min to allow maximal complex formation. Then nonlabeled uv-irradiated T7 DNA, containing a 50-fold greater concentration of dipyrimidines than that of the labeled DNA, is added and aliquots of the mixture are filtered at intervals. The rate of dissociation of the radioactive complexes, k_2, is calculated directly from eq 4 because under these conditions $k_1[E]_t[S]_t$ is negligible. Integration and rearrangement of eq 4 yield

$$\ln [ES]/[ES]_0 = -k_2t, \therefore k_2 = 1/t_{37\%} \quad (5)$$

Determination of the Action Spectrum for the Photolytic Reaction. The rate of the light-catalyzed breakdown of the complexes is measured at several wavelengths using the membrane binding technique in combination with the procedure outlined by Harm and Rupert (1970b). Irradiated T7 [^{3}H]DNA is complexed with excess enzyme in the dark for 30 min. Then the solution is stirred continuously and illuminated in a 1-cm wide quartz cuvet with light from a Bausch and Lomb grating monochromator (Model 33-86-45-49). Light for the monochromator is provided by a mercury vapor lamp (Phillips SP 500) with a quartz cover. Wavelengths below 305 nm are excluded with a mylar filter (DuPont Gauge 50, type A). Aliquots of the illuminated solution are removed at various times and assayed for the ES remaining after photolysis.

The natural logarithm of the fraction of ES remaining following photolysis is plotted as a function of the photoreactivating light dose. The early part of the curve is extrapolated to the value 0.37ES remaining, the e^{-1} value, and the reciprocal of the dose for that value, equal to k_p ($=k_3/I$), is calculated (Harm and Rupert, 1970b). From k_p, $\epsilon\Phi$ (eq 6;

$$\epsilon\Phi \text{ (l. mol}^{-1}\text{ cm}^{-1}) = 5.2 \times 10^9 \times k_p/\lambda \text{ (nm)} \quad (6)$$

Harm and Rupert, 1970b) is evaluated for each wavelength, where ϵ = the extinction coefficient of the active enzyme–substrate complex and Φ = the quantum yield for the dissociation of the complex.

[3] This treatment is approximate and a more general one is discussed in the Addendum.

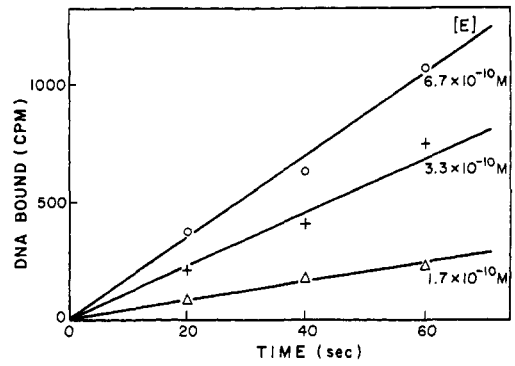

FIGURE 3: Time-dependent ES complex formation. Enzyme, 0.4 ml, was added rapidly in the dark to 0.4 ml of DNA solution I, and the solution mixed on a vortex stirrer. A 0.15-ml aliquot was immediately withdrawn and filtered on an S & S B-6 membrane held in a Millipore microanalysis filter holder (no. XX1002500), an apparatus which allowed almost instantaneous filtration of the sample. The reaction was timed from the moment at which this sample, the zero time control, was filtered to the times at which the other samples were filtered. The "DNA bound" (counts per minute) represents the difference between the counts per minute of the sample and zero time control. The reaction was run at 22° at several different enzyme concentrations, as indicated on the graph.

Results

Determination of Kinetic Constants. Figure 3 is a plot of counts per minute of ES bound as a function of time after mixing photolyase and irradiated T7 [^{3}H]DNA. The average k_1 determined from these plots at three different enzyme concentrations is 2.7×10^7 l. mol^{-1} sec^{-1} at 22° and 1.5×10^7 at 0°. Substituting these values into the Arrhenius equation yields an energy of activation of 4.4 kcal mol^{-1} (Table I). Figure 4 shows the dissociation of ^{3}H-labeled photolyase complexes as a function of time at three different temperatures. For each temperature the t_{37} value is obtained from an extrapolation of the initial linear portion of the curve. Its reciprocal is k_2 (Table I). The complexes are not affected by the presence of unirradiated T7 DNA (Figure 4). An Arrhenius plot of $1/T_{0_k}$ *vs.* log k_2 yields an activation energy of 6.9 kcal mol^{-1}. In Figure 5 there are two plots of $\epsilon\Phi$ *vs.* wavelength, one based on the transformation assay and the other on the binding assay. In each case the curve passes through a maximum at 366 nm.

Effect of pH and Ionic Strength on the Equilibrium of the Complex. Mixtures of photolyase and irradiated T7 [^{3}H]DNA

TABLE I: Summary of Kinetic Constants for Photolyase Based on Binding and Transformation Measurements (Harm and Rupert, 1970a,b).[a]

Constant	Temp (°C)	Binding Assay	Transformation Assay
k_1 (l. mol^{-1} sec^{-1})	0	1.5×10^7	0.8×10^7
	22	2.7×10^7	2.6×10^7
$E_{A(k_1)}$ (kcal mol^{-1})		4.4	9.3
k_2 (sec^{-1})	0	3.3×10^{-3}	2.8×10^{-3}
	22	1.4×10^{-3}	1.8×10^{-3} (23°)
	30	5.6×10^{-4}	7.0×10^{-4}
$E_{A(k_2)}$ (kcal mol^{-1})		6.9	5.4

[a] k_1 and k_2 listed for the transformation assay at 0° are extrapolated values obtained from the original data (H. Harm, personal communication).

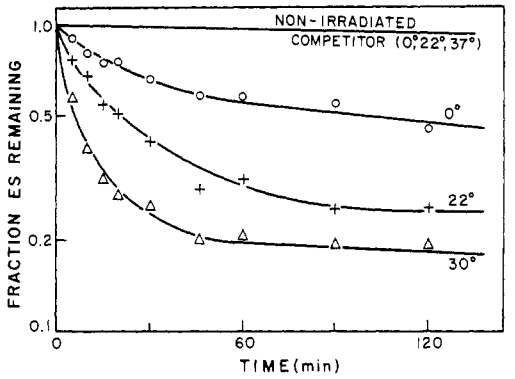

FIGURE 4: Time-dependent dissociation of ES complex. Enzyme, 0.75 ml, and 0.75 ml of DNA solution I were mixed and incubated for 5 min at 22°. Then 0.1 ml of T7 DNA, 18.75 μg/ml (50 J/m^2), was added as the competitor DNA, and a 0.15-ml aliquot was immediately removed and filtered using the Millipore filter holder (see legend to Figure 3). This sample served as the zero-time control, and the counts per minute bound represented the maximal level of complex formation. Samples were withdrawn and filtered at various times relative to the zero-time control. From the counts obtained the fraction of ES remaining was calculated. A control (—) was run in which nonirradiated T7 DNA was substituted for the competitor T7 DNA.

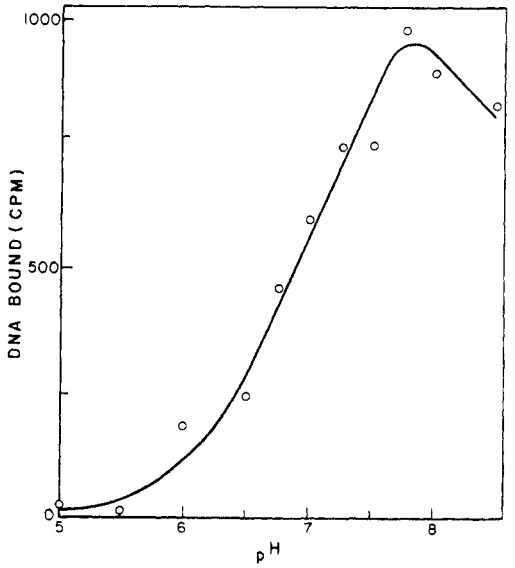

FIGURE 6: Effect of pH on the equilibrium of the ES complex. Two solutions of EDB, containing either 0.05 M KH_2PO_4 (pH 4.9) or 0.05 M K_2HPO_4 (pH 8.4), and 1 mM EDTA, 1 mM HSEtOH, 0.1% bovine serum albumin, and 5% Me_2SO, were titrated against one another to produce several EDB solutions of varying pH values and constant ionic strength. Photolyase was diluted into each of these solutions. Each enzyme solution, 0.1 ml, was mixed with 0.1 ml of tritiated, irradiated DNA solution I, equilibrated for 5 min at 23°, filtered in the dark, and counted.

are prepared having constant ionic strength but varying pH values between 5 and 9, and aliquots are filtered in the dark. Figure 6 shows that maximum binding occurs between pH 7.6 and 7.8.

In other experiments the ionic strength of the reaction mixture is varied by changing the concentration of sodium chloride in the DNA solution, while the pH is held constant. Maximum binding occurs at a μ of 0.175 (Figure 7). When potassium chloride is substituted for sodium chloride, the results are the same.

Sulfhydryl Group Involvement in the Binding of Photolyase to Substrate. Previous work on yeast photolyase has implicated sulfhydryl groups in the mechanism of action of the enzyme. Minato and Werbin (1971) reported that potassium ferricyanide inactivates the enzyme, and that this inhibition can be reversed by dialyzing the enzyme against 2-mercaptoethanol. These findings are based on transformation assays which do not differentiate whether modification of the enzyme affects the light or dark reaction steps. The data in Table II show that ferricyanide inhibits complex formation and that this effect can be partially reversed by dialysis of the ferricyanide-treated enzyme against 2-mercaptoethanol. The data in Table III indicate that reagents that react with SH groups markedly interfere with the photolyase binding step, while those known to cleave S–S linkages are ineffective in inhibiting

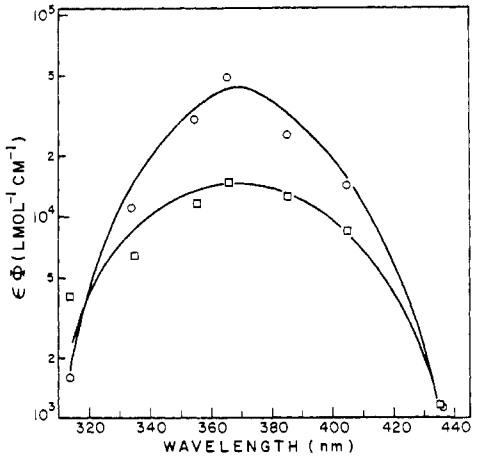

FIGURE 5: Action spectrum for photolyase. DNA solution I, 0.55 ml, was mixed in the dark with 0.55 ml of 2.7×10^{-9} M photolyase, a concentration 5× greater than that of the dipyrimidines in the DNA solution, and held at 23° for at least 30 min. A 0.1-ml aliquot was removed and filtered; the counts per minute of T7 [^{3}H]DNA obtained represented maximal complex formation. The mixture remaining was placed in a 1-cm quartz cuvet and irradiated with monochromatic light. Samples, 0.1 ml, were removed and filtered at intervals. Data obtained from plots of the fraction of complexes remaining *vs.* dose for several wavelengths permitted calculation of $\epsilon\Phi$ by the method of Harm and Rupert (1970b). Action spectra obtained from binding data (○) and from transformation data (□).

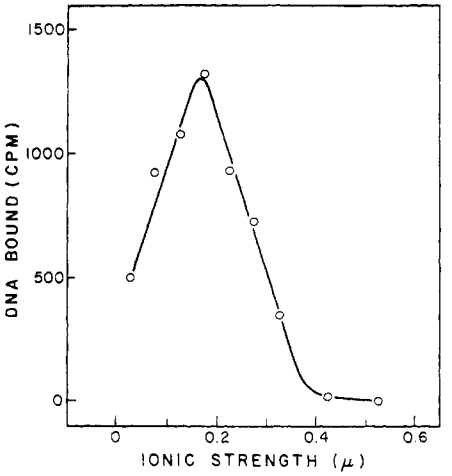

FIGURE 7: Effect of ionic strength on the equilibrium of the ES complex. DNA solutions (containing both irradiated T7 [^{3}H]DNA and calf thymus DNA) were prepared with varying KCl or NaCl concentrations. To 0.2 ml of each there was added 0.2 ml of enzyme in EDB (pH 7.5). After incubation for 5 min at 23°, 0.15-ml replicates were filtered and the membranes counted.

TABLE II: Effect of Ferricyanide on Photolyase Binding.[a]

Potassium Ferricyanide	Dialysis: Against EDB (Minus HSEtOH)	Dialysis: Against EDB (Plus HSEtOH)	% Binding Act.
			(100)
	+		97
		+	90
+			26
+	+		36
+		+	61

[a] Concentrated enzyme was diluted with an equal volume of EDB (lacking HSEtOH) containing 2 mM potassium ferricyanide and incubated for 1 hr at 0°. Aliquots of treated and untreated enzymes (also diluted with EDB) were dialyzed against EDB with or without HSEtOH overnight at 4°. For assay the enzyme was diluted $^1/_{200}$ with EDB.

this reaction. Fe^{2+} and Mn^{2+} reduce binding, while Mg^{2+} does not.

Discussion

DNA photolyase forms a relatively stable complex in the dark with dipyrimidines in far-uv irradiated DNA. In the past the formation and dissociation of these complexes have been followed by the cumbersome techniques of sucrose gradient sedimentation and gel filtration (Rupert, 1962a,b) or indirectly by flash photolysis. On the other hand the membrane binding technique, in which radioactively labeled complexes are trapped on a nitrocellulose membrane during filtration, provides a rapid, convenient, and direct way to determine the kinetic parameters associated with complex formation. This method has also been used to measure several other specific protein–DNA interactions such as the *lac* and λ repressors with their respective operators (Riggs *et al.*, 1970; Chadwick *et al.*, 1970) and P-8 protein from cultured mouse 3T6 cells with single-stranded DNA (Tsai and Green, 1973).

TABLE III: Effect of Various Reagents on Photolyase Binding.[a]

Reagent	Concn (M)	% Binding Act.
None		(100)
Sodium sulfite	10^{-3}	100
	10^{-5}	121
Sodium dithionite	10^{-3}	75
	10^{-5}	87
o-Iodosobenzoic acid	10^{-3}	9
	10^{-5}	61
p-Chloromercuribenzoic acid	10^{-3}	2
Ferrous sulfate	2×10^{-3}	36
Manganese chloride	2×10^{-3}	26
Magnesium chloride	2×10^{-3}	100

[a] Enzyme was diluted with EDB in which one of several reagents was dissolved. The mixture was incubated for 1 hr (under room fluorescent lighting) at 0°. The binding activity at 23° of a control enzyme diluted with EDB, lacking reagent, was set equal to 100%. When a reagent was used, activity was expressed relative to that for the control.

The membrane binding assay depends on the assumption that the efficiency with which the complexes bind to the membranes can be quantitated. At a concentration of one photolyase–dipyrimidine complex/DNA molecule, obtained by varying either the uv dose or enzyme concentration, the efficiency of binding is 20–25% (Madden *et al.*, 1973). This value compares with 30–40% for the *lac* system and 20% for RNA polymerase–DNA complexes (Freeman and Jones, 1967). The per cent efficiency does not vary if the rate of filtration is carefully controlled, and all other experimental parameters are held constant. Therefore, in calculating total enzyme and complex concentrations, an efficiency factor is used to normalize the binding to 100% (eq 3) in the manner of Riggs *et al.* (1970).

In Madden *et al.* (1973) curves of 1/dipyrimidine concentration against 1/DNA bound are plotted for several enzyme dilutions. The one-half saturation point for each of these curves occurs at 2.6 dipyrimidines/DNA molecule, indicating that 5.2 enzyme–dipyrimidine complexes/DNA molecule completely bind the DNA to the filter. We assume that each dipyrimidine binds one enzyme molecule, and on the basis of this assumption the total enzyme concentration is calculated from binding assay data. The agreement between the value obtained and the concentration determined by the flash photolysis–transformation procedure (Harm and Rupert, 1970a) justifies this assumption.

Calculation of k_1 and k_2 by binding measurements yields values in excellent agreement with those obtained by flash photolysis–transformation assay techniques (Table I). The values of k_1 at 0°, determined by the two methods, differ significantly and this leads to divergent values for the calculated energies of activation, 4.4 and 9.3 kcal mol^{-1}. This disagreement is probably due to the technical difficulties of measuring k_1 at 0°.

Riggs *et al.* (1970) report values of k_1 and k_2 at 23° for the *lac* repressor system of 7.0×10^9 l. mol^{-1} sec^{-1} and 6.2×10^{-4} sec^{-1}, respectively. The equilibrium dissociation constant for the *lac* system, K_d ($= k_2/k_1$), is 9×10^{-14} mol $l.^{-1}$, while for photolyase $K_d = 5 \times 10^{-11}$ mol $l.^{-1}$. Therefore, the *lac* repressor–operator complex is 550-fold more stable under optimal conditions than the photolyase–dipyrimidine complex.

When the photolytic constants, $\epsilon\Phi$, are calculated from transforming and binding data, both action spectra show maxima at 366 nm. Differences in the $\epsilon\Phi$ levels arise because it is not possible to use identical experimental conditions with the two methods during photolysis. However, when the transformation assay is run under conditions approximating those of the binding assay, comparable values for $\epsilon\Phi$ are obtained (Harm and Rupert, 1970b).

Formation of photolyase complexes is at a maximum between pH 7.6 and 7.8. Muhammed (1966) found maximal activity at pH 7.2 for partially purified yeast photolyase using the transformation bioassay. Recently, Harm and Rupert (1970a) measured the effects of pH on the rates of association, k_1, and dissociation, k_2, of photolyase complexes. Both constants increased over the pH range 6.0–7.7, but the ratio k_1/k_2 was higher at the lower pH values. We have assumed, as have Riggs *et al.* (1970), that changes in the pH of the filtered solutions have no effect, or only marginal ones, on the efficiency with which complexes bind to membranes.

Riggs *et al.* (1970) observed a 100-fold decrease in the dissociation constant of *lac* repressor–operator complexes when ionic strength was increased tenfold, which they attributed to

strong electrostatic interaction between the protein and DNA. In the case of photolyase complexes, increasing the ionic strength between 0 and 0.175 enhances their formation, but a further increase reduces their number. The reduction at high ionic strength probably reflects interference with electrostatic interactions while the reduction that occurs at lower salt concentrations may be attributed to partial melting of the DNA and the lesser efficiency of photolyase complexation with the dipyrimidines in single-stranded DNA (Setlow, 1964).

Saito and Werbin (1970) showed that photolyase from the blue-green alga *Anacystis nidulans* is inactivated by several agents known to react with protein SH groups. Enzyme inactivated by *o*-iodosobenzoate or ferricyanide could be fully reactivated by reducing agents such as 2-mercaptoethanol, glutathione, sodium hydrosulfite, and sodium bisulfite. Enzyme activity was followed by the transformation bioassay which did not distinguish the dark binding reaction from the photolytic step. Using this assay, similar observations were made with the yeast enzyme (Minato and Werbin, 1971). The membrane binding technique has made it possible to pinpoint the action of ferricyanide on interference with complex formation (Table II). The data differ from that observed previously in the extent of reactivation by reducing reagents, but the reason for this difference is not obvious.

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Addendum

One of the reviewers has brought to our attention that eq 3, dealing with a quantitative treatment of the binding efficiency of the complexes, is an approximation which is exact only for the case in which every DNA molecule has five dipyrimidine binding sites. This distinction becomes significant as saturation of the binding sites is approached because then the efficiency of binding for each DNA molecule is not constant as our approximation implies.

The binding sites are distributed in a manner best defined by Poisson statistics, such that $P(n) = x^n e^{-x}/n!$, where $P(n)$ is the probability of a molecule having n dipyrimidines and x is the average number of dipyrimidines per genome, *i.e.*, five in our system. From this equation the probability (p) of a given DNA molecule binding to the membrane at saturating concentrations of enzyme is given by $p = \Sigma f x^n e^{-x}/(n-1)!$, where f is the probability that a DNA molecule with a single enzyme–dipyrimidine complex will bind to the membrane, *i.e.*, 20% for photolyase. From our approximation that binding is directly proportional to complex formation, it follows that $p = fx = 100\%$, a value in poor agreement with that obtained from Poisson statistics. However, below about 80% of saturation, values obtained by eq 3 approach those obtained by use of Poisson statistics, and it is within this region that our studies were performed. If f is small and x is large, our treatment is accurate, but when f and x approach 1, Poisson statistics must be used.

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