ULTRAVIOLET INACTIVATION DICHROIC RATIO OF ORIENTED fd BACTERIOPHAGE

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ABSTRACT Polarized UV light irradiation of flow-oriented fd bacteriophage indicates that the degree of damage (inactivation) depends on the relative orientation of the light polarization vector and the plane of the DNA bases. The technique of anisotropic UV inactivation was evaluated, and further information on the orientation in this virus was gained. The fd bacteriophage were aligned and irradiated with plane-polarized monochromatic UV light either parallel or perpendicular to the virus axis. Variation of the inactivation dichroic ratio with wavelength implicated virus inactivation by light absorbed in both the DNA and protein. Analysis of the wavelength variation of inactivation dichroic ratios gave molecular dichroic ratios of 0.76 and 1.48 for the DNA and protein components, respectively. On the basis of these anisotropic inactivation studies, the average angle of DNA base tilt in fd was calculated to be 29–32°, a value in agreement with the absorption dichroism studies of Bendet and Mayfield.

INTRODUCTION

The absorption of polarized UV radiation by some biological units depends on the orientation of the plane of polarization of incident light (Jaffe, 1956; Inoue and Sato, 1962; Jaffe and Etzold, 1962). This absorption anisotropy or dichroism[1] can arise from a symmetrical DNA distribution within biological units. Oriented fibers of DNA absorb about three times more polarized 260 nm UV radiation with the electric vector perpendicular to the long axis of the molecule than with the electric vector parallel to the molecular axis (Gray and Rubenstein, 1968). Since different amounts of energy are absorbed by the DNA molecule, it is reasonable to expect some biological function to be preferentially altered, that is, an inactivation dichroic anisotropy might exist in addition to the over-all absorption anisotropy (Deering, Kraus, Gray, and Kilkson, 1966). DNA, besides being a good example

[1] Dichroism (D) is defined as $S_{\parallel} - S_{\perp}$ and the dichroic ratio (R) as $S_{\parallel}/S_{\perp}$, where $S_{\parallel}$ and $S_{\perp}$ are the absorption cross-sections for light polarized parallel and perpendicular to the optic axis of a uniaxial system.

of an UV anisotropic absorber, is also a very important site of UV action in biological systems (Setlow, 1964, 1966). Results of studies on the effects of UV on biological systems implicate DNA as the major target molecule responsible for inactivation of viruses and cells.

Experimentally, the dichroic ratio of any particular cellular component, measured without interference of surrounding components, provides valuable information on its structure and configuration. Deering et al. (1966) have listed the expected dichroic ratio from each of three simple DNA configurations. In determining the dichroic ratio of a particular component, the inevitable problem results from anisotropic absorption measurements being masked by isotropic absorption occurring in other components; therefore, the true anisotropy may not be detected by absorption measurements with polarized light. When DNA is the critical site for UV action, an inactivation anisotropy should be related to DNA structure. Inactivation anisotropies obtained by irradiating bacterial viruses with polarized UV light should give more reliable information on DNA orientation than absorption dichroism measurements, provided that light absorbed in the DNA is responsible for inactivation.

Inactivation anisotropies for intact biological units have not been previously demonstrated (Gray, Kilkson, and Deering, 1965). One way to observe such an inactivation anisotropy is to orient the units in question, to irradiate with plane-polarized light and to compare the survival response for light polarized in different directions relative to alignment of the spatial axes. We report here inactivation anisotropies obtained for just such experiments using the filamentous (870 × 5.6 nm), single-stranded DNA, bacteriophage fd (see review by Marvin and Hohn, 1969).

MATERIALS AND METHODS

Viruses and Cells

ϕX-174 bacteriophages, used for biological actinometry, were obtained originally from Dr. R. L. Sinsheimer, California Institute of Technology, Pasadena, Calif. *Escherichia coli* C (BTCC No. 122) was the host for ϕX-174 assay. The filamentous bacteriophage fd was obtained from Dr. D. A. Marvin of the Department of Molecular Biophysics, Yale University, New Haven, Conn., who initially isolated the phage (Marvin and Hoffman-Berling, 1963). It was grown on *E. coli* K-12 Hfr 3300/1, also obtained from Marvin.

Tryptone broth (containing per liter of distilled water 10 g Difco Bactotryptone, 1 g Difco yeast extract, [Difco Laboratories, Detroit, Mich.] 8 g NaCl, 1 g glucose, 0.5 g Tris, plus 2 ml sterile 1 M $CaCl_2$ added after autoclaving) was used for growing all bacterial cultures.

Phage Preparation

The fd phage were prepared essentially as described by Marvin and Schaller (1966). ϕX-174 phage were prepared as described by Hutchinson and Sinsheimer (1966). Phage stocks were

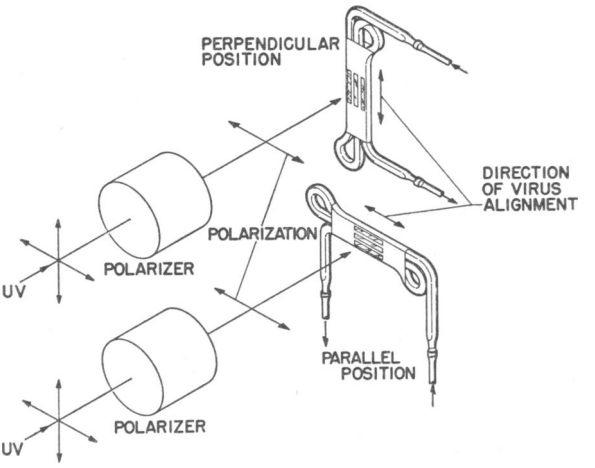

FIGURE 1 Polarization and alignment directions.

kept in sterile 0.08 M $Na_2B_4O_7$ (pH 8.5) which had been twice filtered through a well-rinsed Millipore filter (0.22 μ pore size; Millipore Filter Corp., Bedford, Mass.).

Plating was done using the double-layer technique (Adams, 1959). Tryptone broth top agar (0.7%) and bottom agar (1.0%) were used in the assay procedure.

Dichroism Apparatus

Laminar flow in a 0.41 mm diameter quartz capillary (Thermal American Fused Quartz Co., Montville, N. J.) was used to align the rod-shaped virus. After bending the capillary back upon itself three times to increase the capillary area within the UV beam, the front and back surfaces were ground parallel to one another and polished as shown in Fig. 1.

A flow-regulating system maintained continuous flow through the quartz capillary into a small reservoir which was open to the atmosphere. A variable speed, modified Buchler pump (Buchler Instruments, Inc., Fort Lee, N. J.) returned the solution from the open reservoir to the capillary flow cell. In all experiments, except as specified, flow gradients at the capillary wall were calculated to be 3.9×10^4 sec^{-1}, which corresponds to an average flow rate through the capillary of 16 ml/min. The volume of the entire flow system was about 0.8 ml. Formulation S-50-HL Tygon tubing (The U. S. Stoneware Co., Akron, Ohio) was rinsed for 12 hr with a 10% solution of Tween 80 followed by a 10 min rinse with distilled water before installation in the polystaltic pump. This procedure increased from 15 min to 4½ hr, the time over which 100% phage viability (ϕX-174 and fd) was maintained in control experiments. All experiments were completed in 4 hr or less.

UV Irradiations

All UV irradiations were done with a water-prism monochromator of a design similar to that described by Fluke and Setlow (1954). Sample geometry and variation in UV intensity necessitated using biological actinometry to determine the integral UV dose received by fd. ϕX-174 was selected as a biological dosimeter, since its spherical shape results in non-preferential orientation and it can be mixed directly with the fd phage sample and both ir-

radiated simultaneously. Mixed plating experiments have shown no plaque formation when one virus was plated on the opposite host.

ϕX-174 standard actinometry calibration curves were obtained by irradiating 10^7–10^8 plaque-forming units (pfu) per ml in a 1.0 cm quartz cuvette with continuous stirring. UV intensities were measured with a photocell, the output of which was measured by a Hewlett-Packard Vacuum Tube Voltmeter (VTVM) (Hewlett-Packard Co., Palo Alto, Calif.).

A semiconvex quartz lens was used in conjunction with a 3.0 cm Glan-type (type A) polarizing prism (Karl Lambrecht, D/B/A Crystal Optics, Chicago, Ill.) to collimate and polarize the UV beam, respectively. The UV light beam was 99% plane polarized. The flow cell was rotated to orient the direction of virus alignment either parallel or perpendicular to the plane of polarization (see Fig. 1). ϕX-174 actinometry was repeated for both flow cell orientations and for each experiment. Average intensity inside the capillary at 265 nm (as determined from ϕX-174 actinometry) was about 5.0 ergs/mm^2·min.

For flow cell irradiations, the two virus stocks were mixed and diluted in 0.08 M $Na_2B_4O_7$ to give 10^7 and 10^8 pfu/ml. Duplicate 0.01-ml bacteriophage samples were removed from the open reservoir with a 50 μl Hamilton (No. 705) syringe (Hamilton Co., Whittier, Calif.) after various times of irradiation, diluted in tryptone broth, and plated on agar plates using the appropriate bacterial host. Both phage samples were plated in triplicate at each of two different concentrations. The final sample in every experiment received a sufficient dose such that the 0.01 ml phage samples were plated directly onto their respective hosts without first passing through a dilution series.

EXPERIMENTAL RESULTS

Evaluation of the Flow System

Both viruses were insensitive to inactivation by the high velocity gradient used in these experiments. To check the flow system for uniform sample mixing and

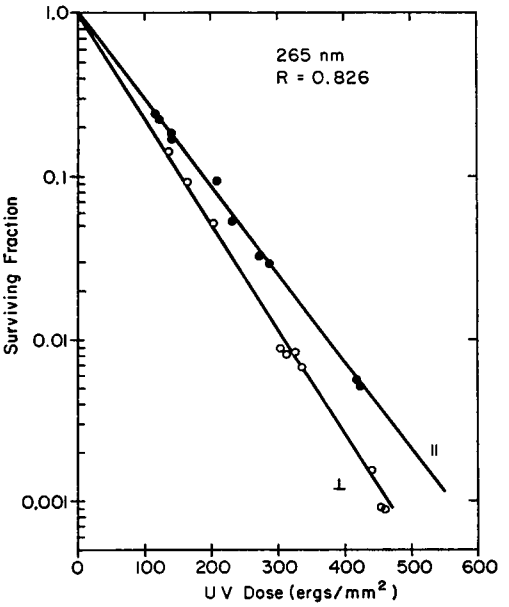

FIGURE 2 Polarized UV inactivation of aligned fd (results from several experiments). The inactivating wavelength was 265 nm with the electric vector either parallel (||) or perpendicular ($\perp$) to the virus axis. Velocity gradient G = 39,100 sec^{-1}.

virus survival, a UV survival curve for ϕX-174 was run as a function of time. No significant deviation from exponential inactivation was observed until very low survival ratios ($N/N_0 = 0.0001$) were obtained.

Inactivation Dichroic Ratio

The survival of fd when exposed to plane-polarized light (265 nm) with the electric vector parallel or perpendicular to the virus axis is shown in Fig. 2. The inactivation dichroic ratio ($\sigma_\parallel/\sigma_\perp = D_{0.01\perp}/D_{0.01\parallel}$, where $\sigma_\parallel$ and $\sigma_\perp$ are the inactivation cross-sections for light polarized with its electric vector parallel and perpendicular, respectively, to the virus axis) was determined directly from Fig. 2 by taking the ratio of the UV dose necessary to reduce the surviving fraction to 0.01 for light

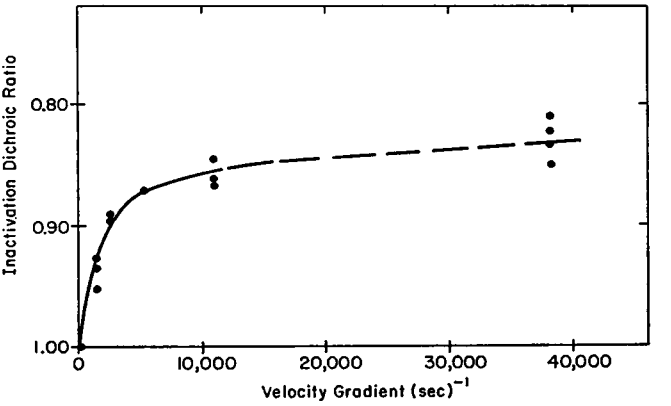

FIGURE 3 Inactivation dichroic ratio (265 nm) as a function of maximum velocity gradient.

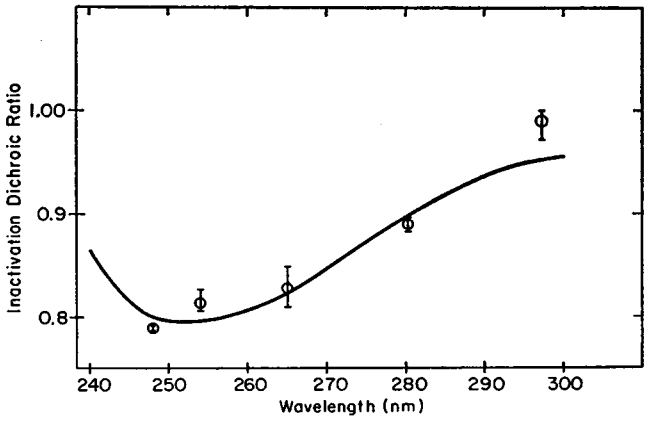

FIGURE 4 Inactivation dichroic ratios measured (points) and calculated to fit (line) as a function of wavelength for aligned fd. Each point is the average of several experiments. The maximum and minimum values at each wavelength are indicated by the vertical lines. Velocity gradient $G = 39{,}100$ sec^{-1}.

polarized perpendicular to the virus axis to the analogous value for light polarized parallel to the virus axis. Numerical values from several experiments varied from 0.81 to 0.85; the average value was 0.83.

The effect of velocity gradient on inactivation dichroic ratio was determined for different values of flow gradient (G), as shown in Fig. 3. No inactivation anisotropy was observed when the flow rate was insufficient to give the viruses a preferential orientation.

The inactivation dichroic ratio was measured at several wavelengths in the range where sufficient UV intensity was available. These results, measured using a flow gradient of 3.9×10^4 sec^{-1}, are plotted in Fig. 4. The magnitude of the inactivation dichroic ratio varied with wavelength, going from 0.79 at 248 nm to 0.99 at 297 nm.

INTERPRETATION AND DISCUSSION

In the wavelength region from 245 to 290 nm, the absorption dichroic ratio for DNA fibers is constant (Gray and Rubenstein, 1968). The dichroic ratio being constant with respect to wavelength implies that, if UV light absorbed only in DNA of fd was responsible for inactivation, then the inactivation dichroic ratio would be expected to remain constant over the wavelength range of Fig. 4. The inactivation dichroic ratio for fd bacteriophage does not remain constant as was anticipated. The increase at longer wavelengths is attributed to phage inactivation resulting from UV light absorbed by the protein coat. This result made it necessary to resolve the inactivation dichroic ratio into a protein dichroic ratio and a DNA dichroic ratio.

The probabilities that a photon absorbed in protein or DNA would cause virus inactivation were estimated as follows. These probabilities are quantum yields for protein (P) and DNA (D) contributions to virus inactivation: Φ_P and Φ_D, respectively. The total virus inactivation cross-section (σ_T) for fd bacteriophage (measured by Rauth, 1965) can be expressed in terms of protein and DNA quantum yields as follows:

$$\sigma_T(\lambda) = \sigma_P(\lambda) + \sigma_D(\lambda)$$

$$\sigma_T(\lambda) = \Phi_P S_P(\lambda) + \Phi_D S_D(\lambda), \quad (1)$$

where S_P and S_D are the absorption cross-sections for fd protein (Knippers and Hoffman-Berling, 1966) and fd DNA (Hoffman-Berling, Marvin, and Dürwald, 1963). The two inactivation quantum yields (Φ_P and Φ_D) were estimated by simultaneously solving equation 1 at several sets of two wavelengths. The estimated values of Φ_P and Φ_D arrived at by this procedure were substituted back into equation 1 at all wavelengths, and the resulting calculated action spectrum was compared to Rauth's measured action spectrum. The best over-all fit by observation resulted when Φ_P and Φ_D were adjusted to 2.15×10^{-3} and 6.5×10^{-3}, respectively. Both of these values were assumed to be independent of wavelength (see Setlow and

TABLE I

MOLECULAR DICHROIC RATIOS FOR fd DNA AND fd PROTEIN

	Molecular dichroic ratios	
	Calculated from inactivation dichroic ratios	Calculated from absorption dichroism*
DNA	0.76	0.68
Protein	1.48	2.14

* See Bendet and Mayfield (1967).

Doyle, 1954; 1957; Setlow, 1961; McLaren and Shugar, 1964; Braunitzer, Asbeck, Beyreuther, Köhler, and von Wettstein, 1967).

The dichroic contributions from individual components (DNA and protein) are referred to as molecular dichroic ratios. The inactivation dichroic ratio (R^I) measured in these experiments can be expressed in terms of quantum yield (Φ_i), absorption cross-section (S_i) and molecular dichroic ratio (R_i) for each of the ith absorbing viral components. A derivation of this equation is contained in the Appendix of this paper.

$$R^I(\lambda) = \frac{\Phi_P S_P(\lambda) R_P(R_D + 2) + \Phi_D S_D(\lambda) R_D(R_P + 2)}{\Phi_P S_P(\lambda)(R_D + 2) + \Phi_D S_D(\lambda)(R_P + 2)}. \quad (2)$$

Molecular dichroic ratios R_P and R_D were determined by simultaneously solving equation 2 at several sets of two wavelengths, and average values are presented in Table I. Molecular dichroic ratios ($R_P = 1.48$ and $R_D = 0.76$) were substituted into equation 2 at all wavelengths to obtain the inactivation dichroic ratio curve (solid line, Fig. 4). Dichroic ratios determined by this method quantitatively account for the observed results of Fig. 4.

Dichroic ratios determined by Bendet and Mayfield (1967) were calculated from absorption dichroism measurements on aligned fd samples and are also shown in Table I. The theory of Higashi, Kasai, Oosawa, and Wada (1963) was used by Bendet and Mayfield to express total fd absorption dichroism as the sum of absorptions from each kind of chromophore. This treatment led to an estimation of molecular dichroic ratios. Considering that completely different experimental techniques and theoretical treatments were used in arriving at the data in Table I, the agreement between DNA dichroic ratios was considered satisfactory.

Base Plane Orientation

For DNA it was assumed that UV absorption arises from one intense π–π^* transition which lies in the plane of the base and makes an angle α with the virus axis (Mason, 1954; Stewart and Davidson, 1963). The molecular dichroic ratio then

can be expressed as:

$$R = \frac{f\cos^2\alpha + 1/3(1-f)}{1/2(f\sin^2\alpha) + 1/3(1-f)}, \quad (3)$$

where f is the fraction of perfectly oriented viruses (Fraser, 1953).

The round cross-section capillary provides a simple flow pattern that easily allows one to estimate the degree of orientation of viruses from hydrodynamic theory and flow birefringence theory (Scheraga, Edsall, and Gadd, 1951; Tanford, 1961). More than 80% of viruses in the UV beam were aligned to within less than 3° of the capillary axis and 97% within less than 7° of the capillary axis.

The angle between the normal to base plane and virus axis is the angle of base tilt, θ. θ and α are complementary angles only if the angle of base twist is zero or very nearly zero. For double-stranded DNA configurations, the angle of base twist is small, i.e., 5–8° (Langridge, Marvin, Seeds, Wilson, Hooper, Wilkins, and Hamilton, 1960; Marvin, Spencer, Wilkins, and Hamilton, 1961; Fuller, Wilkins, Wilson, and Hamilton, 1965). In substituting their absorption dichroism measurements into equation 3, Bendet and Mayfield (1967) assumed a small angle of twist for single-stranded DNA in fd in order to calculate a 20–30° angle of base tilt.

Assuming an error of $\pm 10\%$ in R_D due to (*a*) experimental variation which can be estimated from Fig. 3 and (*b*) an insufficient velocity gradient to permit measurement of R_∞, the range of α's calculated from equation 3 for $f = 0.8$ was 58–61°. The angle of base tilt, θ (calculated assuming $\theta = [90° - \alpha]$) would be 29–32°. The low molar extinction coefficient per phosphate group (260 nm) for fd (Day, 1969) indicates base stacking. Because DNA is more structured than other molecules, the average angle of base tilt can be considered significant.

It was difficult to make any conclusions about the protein structure, since R_P was mostly indicative of tyrosine and tryptophan aromatic ring orientation. These two amino acids would not be expected to have the same transition angle. The average angle of tilt was calculated to be approximately 48° with respect to the virus axis.

The agreement between the DNA molecular dichroic ratio calculated from inactivation measurements and the DNA molecular dichroic ratio calculated from absorption measurements can be interpreted as indirect evidence that the quantum yield (for one component of virus inactivation) for light polarized parallel to the virus axis must be equal to the quantum yield for light polarized perpendicular to the virus axis.

Inactivation measurements have an advantage in that much more dilute solutions can be used than is necessary for absorption measurements. The geometry of the flow cell used here and the resulting uniform velocity gradient also permit alignment of less asymmetric biological units than is feasible with rectangular flow cells commonly used for absorption dichroism measurements.

A potential further advantage of this technique would be to yield information

about the structure of a sensitive component in a multicomponent system where the sensitive component was entirely responsible for the observed anisotropic effect. In such a case, the absorption dichroism becomes too difficult to analyze in terms of individual components. If more than one component is the target for inactivation, the relative sensitivities (Φ's) of each of the absorbing components must be known.

APPENDIX

To calculate the DNA or protein molecular dichroic ratio from the total inactivation dichroic ratio, the following identity can be written:

$$R^I = \frac{\sigma_\parallel}{\sigma_\perp}.$$

Assuming that the quantum yield for virus inactivation by absorption in protein and DNA components was independent of the plane of polarization,

$$R^I(\lambda) = \frac{\Phi_P S_{\parallel P}(\lambda) + \Phi_D S_{\parallel D}(\lambda)}{\Phi_P S_{\perp P}(\lambda) + \Phi_D S_{\perp D}(\lambda)}, \tag{4}$$

where $S_{\parallel P}$, $S_{\perp P}$, $S_{\parallel D}$, and $S_{\perp D}$ represent the protein (P) and DNA (D) absorption cross-sections for light polarized either parallel ($\parallel$) or perpendicular ($\perp$) to the virus axis.

The average absorption coefficient $S_0 = ([S_x + S_y + S_z]/3) = \frac{1}{3}(S_\parallel) + \frac{2}{3}(S_\perp)$. This equation can be expressed in terms of molecular dichroic ratio of the ith component, $R_i = S_\parallel/S_\perp$ (assuming that R_i is constant with respect to wavelength, see Gray and Rubenstein, 1968);

$$S_0 = \tfrac{1}{3}(S_\perp R_i) + \tfrac{2}{3}(S_\perp). \tag{5}$$

Solving for $S_\perp$,

$$S_\perp = S_0/(\tfrac{1}{3}[R_i] + \tfrac{2}{3}) = 3S_0/(R_i + 2), \tag{6}$$

and from the definition of R_i,

$$S_\parallel = R_i S_\perp = 3S_0 R_i/(R_i + 2). \tag{7}$$

Equations 6 and 7 represent the absorption cross-sections for either DNA or protein, where S_0 is the absorption cross-section of either component for unpolarized light and will be called S_D and S_P. Equation 4 can now be written as:

$$R^I(\lambda) = \frac{\Phi_P S_P(\lambda) R_P(R_D + 2) + \Phi_D S_D(\lambda) R_D(R_P + 2)}{\Phi_P S_P(\lambda)(R_D + 2) + \Phi_D S_D(\lambda)(R_P + 2)}. \tag{8}$$

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