INACTIVATION OF ORIENTED BACTERIA WITH POLARIZED ULTRAVIOLET LIGHT

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ABSTRACT Aligned deoxyribonucleic acid (DNA) molecules exhibit a large absorption anisotropy in the ultraviolet (UV) region of the spectrum (11). Also, the UV action spectra of most bacteria resemble the absorption spectrum of DNA (23), implying that inactivation is directly proportional to the UV absorbed by the bacterial DNA. Hence, the UV sensitivity of aligned uniaxial bacteria might be anisotropic with respect to polarization of the incident UV (17, 19). Any inactivation anisotropy would depend upon the orientation of DNA within the bacteria, as well as upon the alignment of bacteria, and could provide a more sensitive indication of *in vivo* DNA orientation than is presently available using optical methods (5, 12–16). Using an electric field of 3.5×10^6 cycles/second, samples of bacteria of strain LS-301 were aligned in a quartz cell and were irradiated with UV ($\lambda = 2652$ A) polarized perpendicular and parallel to the alignment direction. The resultant survival curves resolved no inactivation anisotropy. This result is interpreted to mean that there was insufficient bacterial DNA alignment to give a detectable anisotropy. The minimum average DNA alignment necessary to have resolved an anisotropy is calculated to be 15 per cent in an axial direction (bases perpendicular to the bacterial axis) or 30 per cent in a radial direction (bases parallel to the bacterial axis).

INTRODUCTION

Aligned nucleic acid molecules exhibit dichroism[1] and birefringence[2] (1-11). These two optical anisotropies have been used to study the orientation of nucleic acid

[1] If $S_\parallel$ and $S_\perp$ are the absorption cross-sections for light polarized parallel and perpendicular to the optic axis of a uniaxial system, then $S_\parallel / S_\perp$ is the dichroic ratio and $S_\parallel - S_\perp$ is the dichroism of the system. Nucleic acids are dichroic in the ultraviolet and infrared portions of the spectrum.
[2] If $n_\parallel$ and $n_\perp$ are the refractive indices for light polarized parallel and perpendicular to the optic axis of a uniaxial system, then $n_\parallel - n_\perp$ is the birefringence of the system.

molecules within biological organisms (5, 12-16). If an organism (or a sample of oriented organisms) contains an optically detectable concentration of nucleic acid, the dichroic ratio and birefringence can be measured directly. If the organism is sufficiently large, variations in these anisotropies within the organism can also be measured. Inoué and Sato (14) studied the nucleic acid orientation in cave cricket sperm by using both optical anisotropies. Irradiation of the sperm head with polarized ultraviolet light (UV) damaged the nucleic acid and caused a loss in birefringence. Due to the UV absorption anisotropy of the nucleic acid, there was a non-uniform birefringence loss and a consequent rotation of the optic axis. The magnitude of this rotation was related to the nucleic acid orientation.

For an organism to be studied with the above optical techniques, an optically detectable concentration of nucleic acid is required in the organism (or in a sample of aligned organisms). Therefore, physical studies of *in vivo* nucleic acid orientation have been limited to organisms such as virus and sperm. The above requirement need not be satisfied if the nucleic acid struucture can be studied using a biological assay. This is possible if a physical anisotropy of the nucleic acid results in a detectable biological anistropy.

The UV absorption anisotropy of deoxyribonucleic acid (DNA) may result in anisotropic UV sensitivity of organisms in a sample (17–19). If the lethal effect of UV on an organism is a function of the total UV absorbed by the DNA, the survival of a sample irradiated with polarized UV depends upon these factors:

(*a*) the dose of inactivating UV;

(*b*) the arrangement of organisms within the sample during irradiation;

(*c*) the direction of polarization of the inactivating UV;

(*d*) the dichroic ratio of DNA;

(*e*) the average orientation of DNA within the organisms during irradiation.

If the first four factors are known for samples irradiated with different directions of polarization, limits can be set on the fifth. Only the first three can be controlled experimentally. Maximum resolution of the DNA orientation in a uniaxial organism is achieved when the conditions for irradiation are as follows (18):

(*a*) the dose is large enough to allow an accurate measurement of the survival curve slope;

(*b*) the organisms are perfectly aligned in the samples and fixed during irradiation;

(*c*) the direction of polarization is parallel to the direction of sample alignment for one survival curve, and perpendicular for another.

It is noteworthy that samples with unaligned but spatially fixed organisms can be used for the study of biological anisotropies to polarized UV (17, 18), although the resolution is quite low. For maximum resolution, the organisms should be aligned. This paper describes experiments on the polarized UV inactivation of aligned bacteria.

MATERIALS AND METHODS

Alignment Method. Rod-shaped bacteria in a liquid suspension can be aligned by means of a high frequency electric field so that the bacterial axes lie parallel to the field direction (20, 21). Bacteria were aligned by this method in a strain-free fused quartz cell (Pyrocell Manufacturing Company, Westwood, New Jersey) (Fig. 1). The bacterial suspension was subjected to a pulsed, alternating electric field (10^3 pulses/second, 10^{-4} seconds/pulse, 3.5×10^6 cycles/second) from an oscillator constructed in the laboratory and a General Radio Company unit pulser (type no. 1217-A).

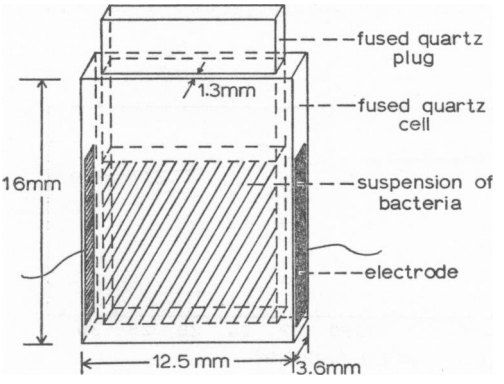

FIGURE 1. Quartz cell. A fused quartz plug was used to reduce any effects caused by the liquid-air interface. The cell volume was 0.1 ml.

Bacteria in sufficient concentration for inactivation could not be aligned in a medium of high conductivity without a loss in viability. At low electric field strengths, alignment was prevented by the excessive field reduction due to charge displacement. At high field strengths, the large cell volume resulted in overheating the sample. (The unfavorable volume to surface ratio did not allow adequate dissipation of heat, heat gain being proportional to the cell volume and heat loss proportional to the surface area.) Therefore, bacteria were aligned in distilled water.

Bacterial Species. The method of alignment required a species of rod-shaped bacteria which remained viable in distilled water. A suitable species was isolated from the laboratory supply of distilled water. The irradiation experiments were carried out with an adenine-requiring mutant which will be referred to as LS-301. A description of this strain is contained in an appendix to this paper.

Bacterial Techniques. An aerated culture of LS-301 was grown to log phase in Difco nutrient broth at 37°C. (Log phase cells were larger than stationary phase cells, giving better alignment and photomicrographic resolution.) The culture was filtered on a 0.45 μ Millipore filter, rinsed with two 100 ml aliquots of distilled water, and washed from the filter with distilled water. A suspension of about 10^8 cells/ml at room temperature was used for the experiments. A comparison of colony counts on nutrient agar (Difco) plates with Petroff-Hausser counts indicated that almost all the bacteria in such a suspension were viable. Viability *versus* time in distilled water is shown in Fig. 2. Although the viability of LS-301 changed slowly with time, there was an initial rapid decrease in UV sensitivity as shown in Fig. 3.

The bacteria were kept in distilled water for a least 12 hours before being irradiated with polarized UV. A 0.1 ml sample was irradiated in the quartz cell for each survival

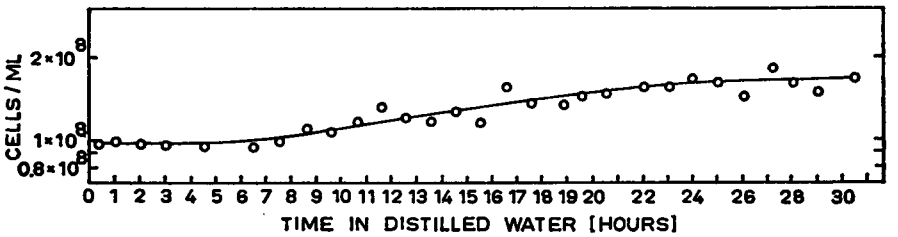

FIGURE 2 Viability of LS-301 in distilled water. For each point, a sample was taken from a distilled water suspension, diluted, plated on nutrient agar plates, and incubated at 37°C.

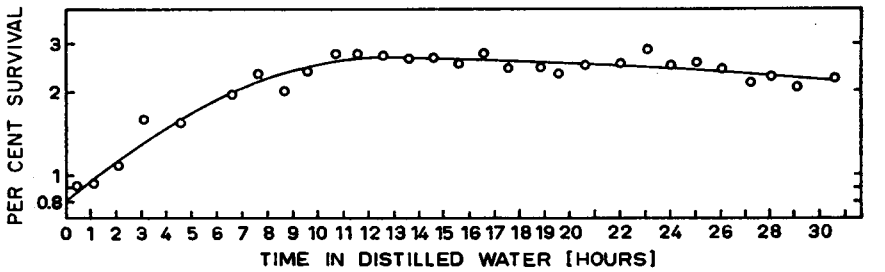

FIGURE 3 UV sensitivity of LS-301 in distilled water. For each point, a sample was taken from a distilled water suspension, diluted, and plated on nutrient agar plates. The plates were irradiated 18 inches away from a 15 watt germicidal lamp and incubated in the dark at 37°C. The dose was the same for each point.

point. The electric field caused no loss of viability in the aligned samples. Temperature rise of the aligned samples due to the electric field was less than 2°C. From each irradiated sample, 0.05 ml was diluted at room temperature in the following medium: KH_2PO_4 — 3.9 gm, K_2HPO_4 — 7.9 gm, $Na_3C_6H_5O_7 \cdot 2H_2O$ — 0.5 gm, $MgSO_4 \cdot 7H_2O$ — 0.1 gm, $(NH_4)_2SO_4$ — 1.0 gm, distilled water — 1000 ml. The bacteria were plated on nutrient agar plates and placed in a dark incubator at 37°C for 24 hours. A Kodak yellow safelight (Wratten Filter Series OA) was the only light used during the postirradiation procedure.

Bacterial Orientation. Photomicrographs of aligned bacteria were taken using a quartz cell with one 0.5 mm face which allowed the use of a 40x objective. Otherwise the cell was identical to that shown in Fig. 1. For the photomicrographs a Leica 35 mm camera was used with a Unitron phase microscope (Model BMPE). The microscope was fitted with a plexiglass stage to avoid disturbing the electric field. Photomicrographs of unoriented and oriented LS-301 are shown in Fig. 4. From such photomicrographs the angular orientation of each bacterial axis was measured with respect to the electric field direction. Some bacteria could not be sufficiently resolved to measure an angle, and they were assumed to be randomly oriented. The resulting angular distribution is given in Fig. 5. Since this distribution did not take into account the rotational symmetry around the electric field axis, a corrected distribution was used in calculating the experimental resolution.

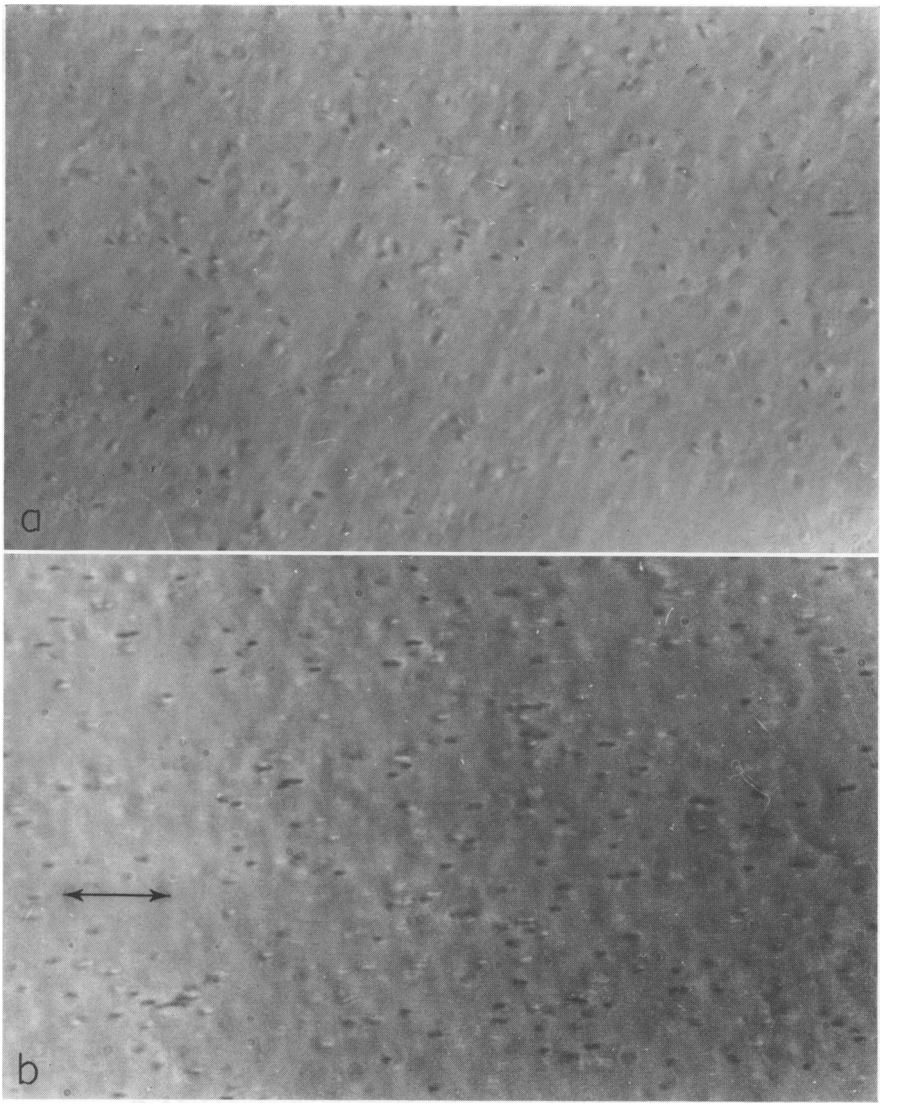

FIGURE 4 Photomicrographs of LS-301 in quartz cell. *a*. No electric field. *b*. Electric field (3.5×10^6 cycles/sec) applied in direction of arrow. Magnification is about 600x. The bacteria appearing white are out of phase contrast.

Irradiation. Irradiations were preformed using a 500 watt high pressure Philips mercury arc with a water prism UV monochromator (22). The 2652 A line was used for the inactivations so as to be near the absorption maximum of DNA. Intensity was measured with a photocell calibrated against an Eppley thermopile. A Glan prism was used as the polarizer. The quartz cell was mounted so that it could be rotated in the beam of polarized UV. Thus, the bacteria could be aligned parallel or perpendicular

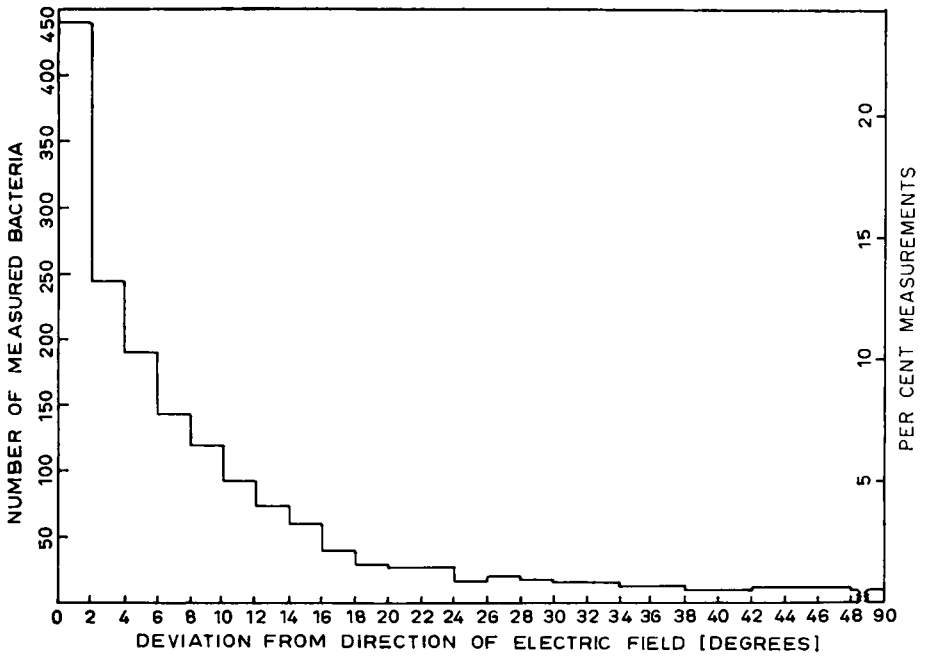

FIGURE 5 Angular distribution of aligned bacteria.

to the direction of polarization, as shown in Fig. 6. There was no optical rotation of the direction of polarization by the bacterial suspension. The transmission was 92 per cent for an unaligned suspension of 10^8 cells/ml. Dose rate was 4.5 erg mm^{-2} $second^{-1}$.

EXPERIMENTAL RESULTS

UV Polarized and Bacteria Unaligned. Survival points for the UV inactivation of unaligned LS-301 are shown in Fig. 7. The incident UV was polarized and the quartz cell placed in the positions shown in Fig. 6, but the electric field was *not applied* to the bacterial suspension during the irradiations. These inactivation controls indicate that the doses received by the cell were equivalent in the parallel and perpendicular positions.

UV Polarized and Bacteria Aligned. Survival points for the UV inactivation of aligned LS-301 are shown in Fig. 8. The incident UV was polarized and the quartz cell placed in the positions shown in Fig. 6. The electric field was applied and the bacteria were aligned during the irradiations.

Interpretation of Experimental Results. No inactivation anisotropy was observed within the experimental resolution. At least two explanations are possible. First, there might be no direct relationship between the survival of the bacteria and the UV energy absorbed by the bacterial DNA. In this case no inactivation anisotropy would be expected, even with perfect alignment of DNA within the

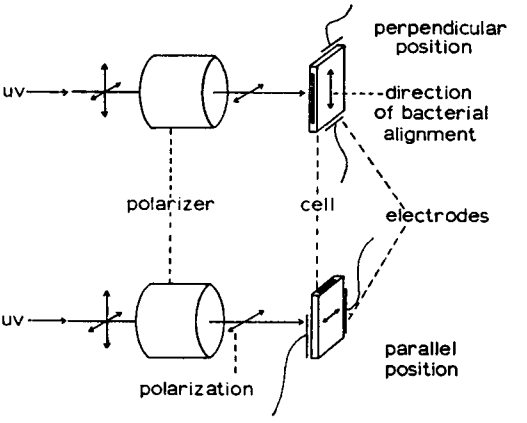

FIGURE 6 Polarization and alignment directions.

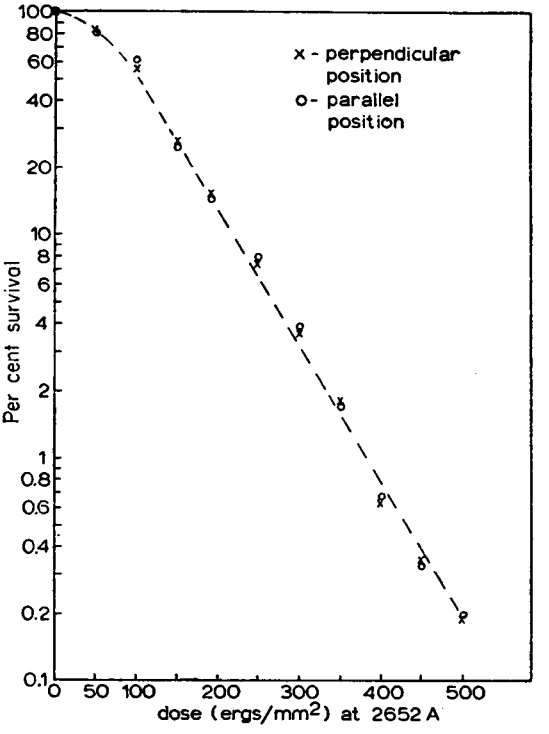

FIGURE 7 UV inactivation of unaligned bacteria.

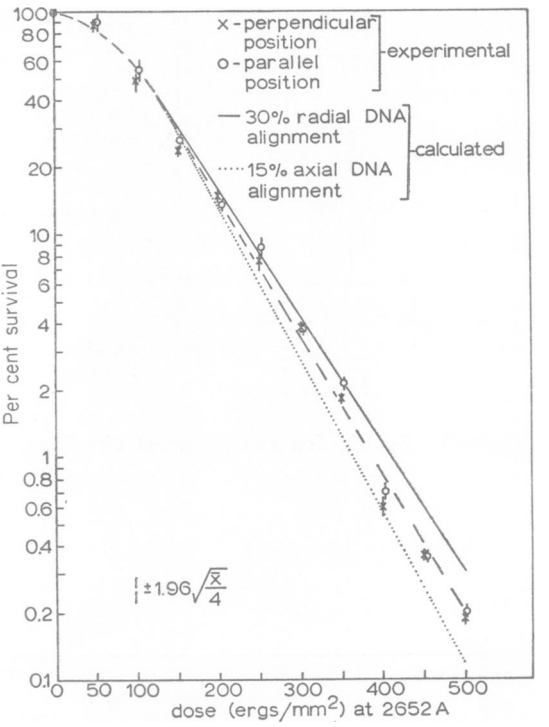

FIGURE 8 Polarized UV inactivation of aligned bacteria. The 95 per cent confidence interval for the mean of the counts from four plates is indicated at each survival point.

organisms. However, the action spectra (relative survival *versus* wavelength of UV) obtained for most bacteria are similar to the absorption spectra of nucleic acids (23). This implies that survival depends upon the amount of energy absorbed by the nucleic acids, regardless of the intermediate photochemistry. It is likely that survival is dependent upon UV absorbed by DNA, rather than by RNA or nucleic acid components, since: (*a*) DNA carries genetic information, and (*b*) there is a close correlation of bacterial inactivation with thymine dimer production and photoreactivation with dimer excision (24, 25). If inactivation is directly proportional to the absorption of UV by DNA, the absorption anisotropy of DNA [$R_{DNA} = S_{\parallel}/S_{\perp} \leq 0.34$ (7, 11)] and DNA alignment should cause UV inactivation which is polarization dependent. Although the dependence of a biological effect on the polarization of UV has not been reported, Inoué and Sato (14) have found that the loss of birefringence in UV-irradiated sperm DNA is dependent on the UV polarization. This effect can be explained in terms of anisotropic damage caused by the preferential absorption of UV in those regions of the sperm DNA which are oriented perpendicular to the polarization. On the basis of these considerations, it

is reasonable to expect an inactivation anisotropy if the DNA is sufficiently aligned within an organism.

A second explanation for the absence of a detectable inactivation anisotropy in LS-301 is that there might not be a sufficient fraction of the DNA aligned within the organisms. As pointed out in the Introduction, the minimum detectable average DNA alignment can be calculated if it is assumed that the inactivation anisotropy of the organisms is directly related to the absorption anisotropy of the DNA. The limits set on the average DNA alignment within the bacteria are largely dependent upon the perfection of bacterial alignment and indicate the resolution obtained in this experiment. These limits are calculated in the following paragraphs where "the average DNA alignment" is understood to include three averaging factors inherent in the experiment:

(*a*) the rotation of each bacterium about its major axis during the irradiation;

(*b*) the various stages of the bacterial life cycle present in the irradiated sample;

(*c*) the fluctuations in DNA orientation within the bacteria during the irradiation.

Polarization-Dependent Inactivation of Aligned Bacteria as a Function of the DNA Alignment. It is assumed that the lethal effect of UV on bacteria is directly proportional to the UV absorbed by the bacterial DNA. The coordinate system used for inactivation of aligned uniaxial bacteria is shown in Fig. 9. The

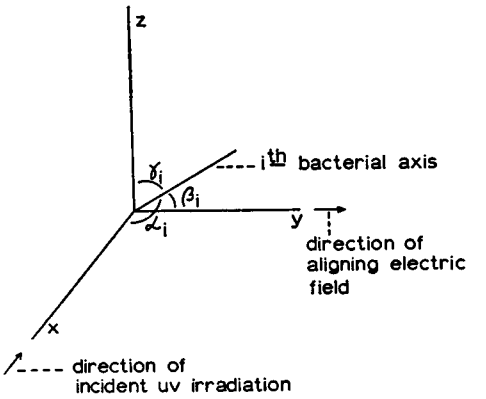

FIGURE 9 Coordinate system for calculation.

aligning electric field is directed along the *y*-axis and the incident UV irradiation is directed along the *x*-axis. Then the parallel and perpendicular directions of UV polarization are along the *y*-axis and *z*-axis, respectively.

Definitions:

$\cos \alpha_i$, $\cos \beta_i$, $\cos \gamma_i$—the direction cosines for the i^{th} bacterium as shown in Fig. 9.

$\overline{\cos^2 \alpha}$, $\overline{\cos^2 \beta}$, $\overline{\cos^2 \gamma}$—the average values for the squares of the direction cosines for n bacteria.

$g(\beta_i)$—the angular distribution function about the alignment direction for n bacteria.

$S_{b\perp}$ [$S_{b\parallel}$]—the absorption cross-section for UV polarized perpendicular [parallel] to the bacterial major axis.

$R_b = S_{b\parallel}/S_{b\perp}$—the bacterial dichroic ratio.

$S_\perp$ [$S_\parallel$]—the absorption cross-section for UV polarized perpendicular [parallel] to the DNA long axis.

$R_{DNA} = S_\parallel/S_\perp$—the dichroic ratio of DNA.

F_a [F_r]—the fraction of the bacterial DNA which is axially [radially] aligned.

D_y [D_z]—the dose of UV given with the direction of polarization along the y-axis [z-axis].

N_y/N_o [N_z/N_o]—the fraction of N_o bacteria which survived the dose D_y [D_z].

ϕ—the quantum efficiency with which the absorbed UV produces a lethal effect.

The values of $\overline{\cos^2 \alpha}$, $\overline{\cos^2 \beta}$, and $\overline{\cos^2 \gamma}$ are determined by $g(\beta_i)$, which can be found experimentally. Since $\cos^2 \alpha_i + \cos^2 \beta_i + \cos^2 \gamma_i = 1$, it follows that

$$\overline{\cos^2 \alpha} + \overline{\cos^2 \beta} + \overline{\cos^2 \gamma} = 1. \quad [1]$$

By definition,

$$\overline{\cos^2 \beta} = (1/n) \sum_{i=1}^{n} g(\beta_i) \cos^2 \beta_i. \quad [2]$$

The symmetry of the angular distribution (Fig. 9) requires $\overline{\cos^2 \alpha} = \overline{\cos^2 \gamma}$. Using equations [1] and [2],

$$\overline{\cos^2 \alpha} = \overline{\cos^2 \gamma} = (1/2)(1 - \overline{\cos^2 \beta}) = (1/2) - (1/2n) \sum_{i=1}^{n} g(\beta_i) \cos^2 \beta_i. \quad [3]$$

The bacterial dichroic ratio R_b is related to the fraction of DNA aligned in the bacteria. Regardless of the exact configuration of the DNA within the bacterial cells, the DNA can be considered as existing in an unaligned fraction and an aligned fraction, where the aligned fraction is either axially aligned (with the DNA axis parallel to the bacterial axis) or radially aligned (with the DNA axis perpendicular to the bacterial axis). The dependence of R_b on the aligned fraction differs in the two cases:

(*a*) If F_a is the fraction of axially aligned DNA,

$$R_b(\text{axial}) = \frac{S_{b\parallel}}{S_{b\perp}} = \frac{F_a S_\parallel + (1 - F_a)[(1/3)S_\parallel + (2/3)S_\perp]}{F_a S_\perp + (1 - F_a)[(1/3)S_\parallel + (2/3)S_\perp]}$$

$$= \frac{R_{DNA}(2F_a + 1) + 2(1 - F_a)}{R_{DNA}(1 - F_a) + (F_a + 2)}. \quad [4]$$

(*b*) If F_r is the fraction of radially aligned DNA,

$$R_b(\text{radial}) = \frac{S_{b\parallel}}{S_{b\perp}} = \frac{F_r S_\perp + (1 - F_r)[(1/3)S_\parallel + (2/3)S_\perp]}{F_r[(1/2)S_\perp + (1/2)S_\parallel] + (1 - F_r)[(1/3)S_\parallel + (2/3)S_\perp]}$$

$$= \frac{2R_{DNA}(1 - F_r) + 2(F_r + 2)}{R_{DNA}(F_r + 2) + (4 - F_r)}. \quad [5]$$

It has been calculated that the survival of aligned bacteria irradiated with polarized UV is given by

$$N_y/N_o = \exp\{-D_y S_{b\perp}\phi - D_y S_{b\perp}\phi(R_b - 1)\overline{\cos^2\beta}\}, \quad [6]$$

or by

$$N_z/N_o = \exp\{-D_z S_{b\perp}\phi - D_z S_{b\perp}\phi(R_b - 1)\overline{\cos^2\gamma}\}, \quad [7]$$

for the direction of polarization along the *y*-axis or the *z*-axis, respectively (18). Although expressions [6] and [7] were derived for one hit inactivations, they can be applied to the straight line portion of multihit inactivation curves. When plotted in a semilogarithmic manner, the ratio of the slopes of equations [6] and [7] is given by:

$$\frac{[\ln(N_y/N_o)]/D_y}{[\ln(N_z/N_o)]/D_z} = \frac{1 + (R_b - 1)\overline{\cos^2\beta}}{1 + (R_b - 1)\overline{\cos^2\gamma}}. \quad [8]$$

From the previous discussion, it is seen that this ratio depends upon the alignment function $[g(\beta_i)]$ and upon the fraction aligned (F_a or F_r), since R_{DNA} is a constant. Hence, values of [8] can be calculated and compared with experimental inactivation curves.

Experimental Resolution. The photographically determined angular distribution (Fig. 5), corrected for rotational symmetry around the alignment direction, is used in equations [2] and [3] to give

$$\overline{\cos^2\beta} = 0.82, \quad [9]$$

and

$$\overline{\cos^2\gamma} = 0.09. \quad [10]$$

Equations [9] and [10] are substituted in [8].

$$\frac{[\ln(N_y/N_o)]/D_y}{[\ln(N_z/N_o)]/D_z} = \frac{1 + (R_b - 1)(0.82)}{1 + (R_b - 1)(0.09)}. \quad [11]$$

Values of [11] are then calculated and compared with the experimental inactivation curves. For example, a value of $F_a = 0.15$ in equation [4] and [11] gives

$$\frac{[\ln(N_y/N_o)]/D_y}{[\ln(N_z/N_o)]/D_z}(\text{axial}) = 0.91. \quad [12]$$

A value of $F_r = 0.30$ in equations [5] and [11] gives

$$\frac{[\ln (N_y/N_o)]/D_y}{[\ln (N_z/N_o)]/D_z} \text{ (radial)} = 1.09. \quad [13]$$

R_{DNA} is taken to be 0.34 (11) in equations [4] and [5].

Equations [12] and [13] show that the slope is greater for the perpendicular polarization when the DNA is axially aligned, but is greater for the parallel polarization when the DNA is radially aligned.

The above equations can now be applied to the inactivation curves of LS-301. Using the slope of the dashed line in Fig. 8 as the value for $[\ln(N_y/N_o)]/D_y$ in equations [12] and [13], the resulting values for $[\ln(N_z/N_o)]/D_z$ gives the dotted and solid lines respectively in Fig. 8. Thus, if there was an average of 15 per cent axial alignment of the bacterial DNA, a change of survival points from the dashed to the dotted line would be expected for a change of polarization from the parallel to the perpendicular position. Likewise, for 30 per cent radial alignment, a change of survival points from the dashed to the solid line would be expected for a change in polarization from the parallel to the perpendicular position. Either change could easily have been resolved experimentally, but no difference in the survival curves was observed. An explanation of this result is that the bacteria LS-301 have an average DNA alignment of less than 15 per cent in an axial direction and less than 30 per cent in a radial direction.

DISCUSSION

Fitting a long bacterial DNA strand (the order of 1000 microns long) into its cell (about 1 × 3 microns) requires that the DNA be compactly arranged. Although there is no *a priori* reason for expecting an arrangement of DNA with a net alignment, such an arrangement is not improbable since electron micrographs indicate structural definition in the bacterial nucleus (26) and DNA is preferentially oriented in virus (13, 15, 27) and sperm (12, 14). An ordered bacterial DNA arrangement might have been detected in the experiments described here, but it was not. Several explanations are possible: (1) There is no inactivation anisotropy even if there is a net absorption anisotropy in the cellular DNA arrangement. However, all available evidence indicates that damage to the DNA, and hence cell inactivation, depends directly on the amount of *absorbed* energy. If there is a preferential absorption direction in the UV sensitive material, there should be a preferential inactivation direction. (2) The net DNA alignment lies within the limits calculated above. The following possibilities for the lack of an average DNA alignment could not be distinguished : (*a*) the cellular DNA does not exist in an orderly arrangement; (*b*) the DNA is regularly ordered but has little or no preferential alignment with respect to the cell axis; (*c*) the position of the DNA structure varies in time within each cell, or varies from cell to cell; (*d*) the DNA has a preferential alignment

only during a small portion of the division cycle. (The cultures used here were not synchronous.)

Even though a cell has no net DNA alignment, short regions of the DNA corresponding to a gene might be oriented with respect to the cell axis, at least during portions of the division cycle. Hence, there exists the possibility for anisotropic production of mutations following irradiation of oriented, synchronous cells with polarized UV. Also, specific cell organelles, as well as cell functions other than gross survival and mutation rate, might be anisotropically altered by polarized UV. Of additional importance would be experiments on systems other than bacteria, such as oriented DNA or virus preparations. These experiments are being planned. It is expected that such approaches will eventually be able to contribute information as to the arrangement of DNA in viruses and cells.

APPENDIX

Some characteristics of the bacterial strain used in the above work are:

Gram-negative

Rod-shaped, approximately 1 $\mu \times 3\ \mu$

Aerobic

Motile

Grows in a medium of the following composition:

KH_2PO_4 – 3.9 gm, K_2HPO_4 – 7.9 gm, $Na_3C_6H_5O_7 \cdot 2H_2O$ – 0.5 gm,

$MgSO_4 \cdot 7H_2O$ – 0.1 gm, $(NH_4)_2SO_4$ – 1.0 gm, glucose – 2.0 gm,

adenine –0.1 gm, distilled water –1000 ml (the original isolate does not require adenine).

Does not ferment glucose, lactose, sucrose, salicin, maltose, mannitol, rhamnose, arabinose, raffinose, inositol, dulcitol, xylose.

Colony type on MacConkey agar: 0.2 mm in diameter, convex, smooth, entire, slightly pink. On blood agar: 1.0 mm in diameter, non-hemolytic.

A distinguishing characteristic of the strain is its ability to live in distilled water.

Further characteristics are contained in Specimen Report No. LS-301 of the Laboratory Division of the Connecticut State Department of Health, Hartford, Connecticut.

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