Action Spectrum for Growth Delay Induced in *Escherichia coli* B/r by Far-Ultraviolet Radiation

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An action spectrum for growth delay induced in *Escherichia coli* B/r by far-ultraviolet radiation (230 to 295 nm) was obtained. It resembles the action spectrum for killing obtained in the same experiments, indicating that the chromophore for growth delay is probably the same as the chromophore for killing. Another action spectrum for killing, obtained under conditions more suitable for chromophore identification, suggests that nucleic acid, either deoxyribonucleic acid or ribonucleic acid, is the chromophore for growth delay induced by far ultraviolet. Isoprenoid quinones, which seem to be important chromophores for growth delay induced by near-ultraviolet radiation (above 300 nm), appear to play a negligible role in growth delay induced by wavelengths below 300 nm.

This paper reports an action spectrum for growth delay induced in *Escherichia coli* B/r by far-ultraviolet radiation (far UV, wavelengths below 300 nm). These experiments were undertaken in an effort to detect a chromophore other than nucleic acid that might operate in far-UV effects on bacteria. Quinones are suspected chromophores for growth delay induced by near-ultraviolet radiation (near UV, wavelengths above 300 nm), and it was felt that they might play a similar role in the far-UV region. The detailed rationale for this work is outlined below.

After ultraviolet (UV) irradiation, some strains of *E. coli*, such as *E. coli* B, continue extensive growth and deoxyribonucleic acid (DNA) synthesis at normal rates in the absence of cell division, i.e., form filaments (1). Some strains, such as *E. coli* 15 T⁻A⁻U⁻, undergo some division and DNA synthesis without growth as they enter the stationary phase (11). Division and growth are, therefore, somewhat separable processes in *E. coli*, and this necessitates operational definitions. In this paper, we use the term "division" to mean cytokinesis resulting in an increase in cell numbers that can be observed either by plating or by Coulter counting. By "growth" we mean mass increase that can be measured by increased turbidity of the suspension.

Near-UV radiation induces extensive growth delay and division delay in *E. coli* at doses pro-

ducing little killing (4, 7, 8). These doses of near UV immediately inhibit respiration and selectively inactivate the isoprenoid quinones (such as vitamin K₂ and coenzyme Q) of the electron-transport chain (8, 9). Furthermore, the action spectra for induction of growth delay (7) and division delay (13) in *E. coli* B are similar to the absorption spectrum of vitamin K₂ in isooctane and to the action spectrum for destruction of vitamin K₂ in isooctane (Jagger and Takebe, Intern. Congr. Photobiology, Hanover, 1968). These findings suggest that isoprenoid quinones may be the chromophores for growth delay and division delay induced by near UV.

In the far-UV region, most of the commonly observed effects are considered to be due to absorption in nucleic acids, and this has been thoroughly demonstrated for cell killing (*see* 5). Far UV, however, also produces growth delay and division delay, and it is conceivable that these effects are partly due to absorption in the quinones. Far-UV induction of *division delay* (filament formation) in *E. coli* B occurs at doses producing little killing, and the action spectrum has been shown to be similar to the absorption spectrum of nucleic acid (1). It therefore seems likely that nucleic acid is the target molecule.

Such an action spectrum has not been obtained, however, for *growth delay* induced by far UV, and it remains possible that there is a quinone contribution to this effect. The absorption spectra of the common isoprenoid quinones are suffi-

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ciently different from that of nucleic acid that action spectra for growth delay in the far-UV region should reveal any significant role of the quinones. Furthermore, we know that in the near-UV region quinones are probably involved but nucleic acids almost certainly are not; therefore, it would be of interest to determine the wavelength region at which there is a switch in the major chromophore for growth delay.

Swenson and Setlow (16) have shown in both *E. coli* B₈₋₁ and *E. coli* B/r that inhibition of DNA synthesis is maximal at 265 nm and is photoreactivable, and Hanawalt and Setlow (3) have shown that the action spectra for inhibition of protein and ribonucleic acid (RNA) synthesis in *E. coli* 15_{T-} and *E. coli* B are similar to the absorption spectrum of nucleic acid. Therefore, in these strains nucleic acid is probably the major chromophore for overall growth delay of the population. However, since extensive killing occurs in all these experiments, it is possible that one is simply observing the action spectrum for killing (and photoreactivation thereof) and that the delays in synthesis observed reflect merely the lag time required for the surviving cells to multiply to the point where their synthetic activities can be observed. In a recent discussion of this point, K. C. Smith said, "We have found that curves [of DNA synthesis] similar to those obtained after irradiation can be generated by diluting unirradiated cells to the same viable count present in irradiated cultures of *E. coli* B/r. Because . . . DNA synthesis kinetics are dependent upon the number of viable cells present [previous authors] have overestimated the duration of UV-induced DNA synthesis inhibition . . ." (Biophys. J. 8:A-51, 1968). It therefore appears that important effects on cell growth, other than those resulting from damaged nucleic acid, could be masked in the presence of extensive cell killing. Such masked effects might be revealed by making corrections for cell killing, and this has been done in the present experiments.

Secondary chromophores for far-UV effects might be more readily revealed by using radiation-resistant organisms, in which much of the DNA damage is repaired. [A very UV-resistant organism, *Micrococcus radiodurans*, has recently been shown to have action spectra for killing and for delay in DNA synthesis that suggest involvement of chromophores other than nucleic acid (15).] *E. coli* B/r was therefore chosen for the present studies because it is relatively resistant to far-UV killing, and is more likely to show effects on growth that are unrelated to killing or to nucleic acid damage. (The amount of growth delay at 80% survival in strain B/r is about four times

that in strains B and 15_{T-}.) Furthermore, the magnitude of killing corrections is minimized. This strain also shows practically no filamentation after irradiation, which simplifies interpretation of turbidity measurements.

MATERIALS AND METHODS

E. coli B/r cells were grown overnight at 37°C with shaking in Difco Nutrient Broth with 0.4% NaCl added. The culture was then diluted 1:100 in fresh medium and again grown for about 2 hr to a concentration of about 1.5×10^8 cells/ml. These cells were then centrifuged, washed, and resuspended in 0.067 M sodium-potassium phosphate buffer, which was used for all later dilutions. The cells were then starved for 1 hr at 37°C. This starvation does not interfere with subsequent growth.

The cells were irradiated at room temperature by far UV from a Bausch & Lomb grating monochromator (no. 33-86-45-49, 10 × 10 cm grating with 1,200 lines per mm), with a Philips SP-500 mercury arc lamp as source. The band width was 10 nm at 230 nm and was usually 3.2 nm or less at other wavelengths. Dose rates were measured with a calibrated thermopile and varied from 1.3 to 17 ergs per mm² per sec. No significant dose-rate dependence was observed. The suspension was irradiated in a quartz spectrophotometer cuvette (1 cm optical path) and was stirred vigorously by a small bar with a magnetic stirrer. The maximal time of exposure for a single wavelength varied from 20 sec to 4 min. The highest dose at each wavelength permitted at least 40% survival.

The optical density of the cell suspension (1.5×10^8 /ml) was 1.45 (3.4% transmittancy) at 260 nm. Such a high optical density is not desirable for determination of action spectra, but was necessary in order to obtain growth curves in which the initial slopes were steep enough to permit early observation of growth delay. Correction of the UV dose for absorption of light by the cell suspension was made according to the method of Morowitz (12); this also corrects partly for scattering.

After exposure, 0.8 ml of the cell suspension was added to 6.5 ml of prewarmed Nutrient Broth in a cylindrical glass cuvette (30 mm path length). The cuvette was then placed in a Bonet-Maury Biospectrophotometer, in which the cells were grown at 37°C with magnetic stirring. The Biospectrophotometer can hold 6 cuvettes and automatically records growth curves (optical density) for each cuvette. Photoreactivation was reduced to an undetectable level by placing, between the light source and the cuvettes, a green filter that absorbs shorter wavelengths (optical density = 1.0 at 435 nm, 2.0 at 410 nm).

To determine viability, samples were taken from the growing culture, diluted in phosphate buffer, plated on Difco Nutrient Agar, and incubated for 24 hr at 37°C. Visible colonies were then counted.

Vitamin K₂ and coenzyme Q₆ were kindly supplied by A. F. Brodie. Absorption spectra of the quinones were measured with a Cary recording spectrophotometer, model 14.

RESULTS

Estimate of the true growth delay after correction for killing and absorption. Figure 1 shows an example of growth curves recorded by the Biospectrophotometer. Increasing the dose of radiation caused increased growth delay. The growth-delay factor is defined as the ratio t_x/t_0 , where t_0 is the time required for the control culture to reach 50% transmittancy and t_x is the time required for the irradiated culture to reach the same transmittancy. Assuming that killed cells do not grow at all from the beginning of postirradiation incubation, the *apparent* delay caused by killed (hence nongrowing) cells could be estimated by use of diluted control cells (Fig. 2 and 3). The *true* (corrected) growth-delay factor, y , for an experiment is calculated by the equation,

$$y = \frac{t_x/t_0}{t_{dx}/t_0} = \frac{t_x}{t_{dx}}$$

where t_{dx} is the time required for a culture, diluted to the survival level of that experiment, to reach 50% transmittancy. A value of t_{dx}/t_0 was obtained from Fig. 3 for each survival level. For example, if the survival is 50%, $t_{dx}/t_0 = 1.32$.

FIG. 1. Typical single experiment showing growth delay of *Escherichia coli* B/r in Nutrient Broth induced by irradiation at 270 nm. The curves were recorded by a Bonet-Maury Biospectrophotometer. Cells irradiated in phosphate buffer were immediately diluted 1:9 into Nutrient Broth (time 0). The dose (ergs/mm²), which is corrected for absorption and scattering by the cells, is shown on each curve. The ordinate shows percentage transmittancy of the culture; t_0 and t_x are defined in the text.

FIG. 2. Growth curves of diluted unirradiated samples, used to estimate the influence of effective reduction in cell number by killing. Numbers on the curves show percentage of initial cell concentration; t_0 and t_{dx} are defined in the text.

FIG. 3. Apparent growth delay caused by an initial reduction in cell number (see Fig. 2). Each point represents an average of six measurements.

Since survival was always above 40%, corrections for killing never involved a factor of more than 1.4.

If irradiation caused only loss of cells (by killing) at the start of incubation, the curves in Fig. 1 would have the same shape and horizontal displacement as those shown in Fig. 2, and no delay would be observed after correction for killing. The fact that horizontal displacements were greater than expected from killing is a reflection of a true growth delay.

Slopes of the curves of the diluted samples at 50% transmittancy decrease only very slightly

with increasing dilution (Fig. 2). Slopes of the curves of the irradiated samples decrease considerably with increasing doses (Fig. 1), and attain a constant slope only much later than those of diluted samples. This suggests that recovery from the growth delay is distributed in the population.

Action spectra for growth delay and killing by far UV. The reciprocal of the true growth-delay factor ($1/y$) was plotted on a logarithmic scale against the dose corrected for absorption and scattering (Fig. 4). The action spectrum for growth delay was calculated by taking the reciprocal of the dose corresponding to $1/y = 0.80$ and correcting this for quantum energy. This dose is needed to increase by a factor of 1.25 the time required to reach 50% transmittancy. All values used for the action spectra are averages of at least three experiments for each wavelength. Results are shown in Fig. 5 (curve A).

Survival data obtained in the growth-delay experiments were used to construct an action spectrum for killing (curve B in Fig. 5). Except for the point at 230 nm, this action spectrum is identical, within experimental error, with the action spectrum for growth delay.

Curve B in Fig. 5 shows higher effectiveness for bacterial killing at long wavelengths than is expected. This result may be biased by the technique used, especially the irradiation of high concentrations of bacteria, which necessitates large corrections for absorption and scatter. We therefore obtained an action spectrum for killing of

FIG. 4. Reciprocal of the true growth-delay factor for each wavelength (nm), plotted on a logarithmic scale against corrected dose (ergs/mm²). At an ordinate value of 0.80, the curve gives the dose needed to increase by a factor of 1.25 the time required to reach 50% transmittancy. This value is used in calculation of the action spectrum with additional quantum corrections. Each curve represents an average of at least three experiments. Open and closed circles are for easier differentiation of the curves. Some of the intermediate wavelengths are not shown.

FIG. 5. Action spectra for growth delay and killing normalized to 100 at the highest point. Curve A (●), for growth delay, was obtained by calculation of the true growth-delay factor from data such as those shown in Fig. 4, and is corrected for killing. Curve B (○), for killing, was obtained by comparing the doses required for 50% survival at each wavelength, using data from the growth-delay experiments, in which cells were irradiated at high concentration. Curve C (△), for killing, was obtained from separate experiments at low cell concentration. Doses for spectra A and B were corrected for absorption and scattering by the cell suspension.

E. coli B/r cells in the same physiological state, but at a concentration (10^8 cells/ml) essentially transparent to all the wavelengths used. In these experiments, we used a constant dose rate of 6 ergs per mm² per sec. This action spectrum (curve C in Fig. 5) shows the expected peak at 265 nm and lesser effectiveness of the longer wavelengths. We believe that this is an accurate action spectrum for killing of *E. coli* B/r and that the spectrum obtained at high concentrations is biased.

Absorption spectra. Figure 6 shows absorption spectra of DNA and of the isoprenoid quinones, vitamin K₂ and coenzyme Q₆. Also shown is the action spectrum for killing of *E. coli* B/r (curve C from Fig. 5), which is assumed to be similar to the action spectrum for growth delay, in view of the similarity of curves A and B of Fig. 5.

The action spectrum for killing (and growth delay) of *E. coli* B/r can be seen to be quite similar to the absorption spectrum of DNA. It is very different from the absorption spectrum of vitamin K₂ (especially in the region of 280 nm) and quite different from the absorption spectrum

FIG. 6. Absorption spectra of vitamin K_2 in iso-octane and coenzyme Q_6 in methanol. The absorption spectrum of DNA, pH 7.0 in phosphate buffer (6), and the action spectrum for killing of *E. coli* B/r (C = curve C in Fig. 5) are shown for comparison. Each curve was adjusted to 100 at the highest point.

of coenzyme Q_6 (especially in the region of 250 nm).

DISCUSSION

The far-UV action spectrum for growth delay (ρ as reflected in curve C of Fig. 5 and 6) differs so greatly from the absorption spectra of the naphthoquinone vitamin K_2 and the benzoquinone coenzyme Q_6 that it may be concluded that the latter are not chromophores for growth delay at wavelengths below 300 nm. The similarity of the action spectrum to the absorption spectrum of DNA (Fig. 6) suggests that nucleic acid is the chromophore in this wavelength region both for growth delay and for killing. (Since the absorption spectrum of RNA is similar to that of DNA, RNA cannot be excluded as a possible chromophore.) Although the fact that the maximum of the action spectrum is at a slightly longer wavelength than the maximum for absorption by DNA might suggest some protein involvement, this seems unlikely in view of the fact that the action spectrum values are actually lower than those for DNA at wavelengths above 280 nm.

In addition, the doses required to induce growth delay and division delay by near UV are of the order of 10^5 ergs/mm² (7), but for far UV they are of the order of 10^2 ergs/mm². However, only approximately a 10-fold difference in quinone absorption in the near and far UV is found (Fig. 6). If the quantum yield for growth delay

does not change greatly with wavelength, then these considerations support our conclusion that growth delay in the far-UV region is not mediated by the same quinone(s) effective in the near-UV region.

Correction for killing was based on the assumption that the killed cells did not grow at all. This might not be correct, because some of the killed cells may be reactivated by liquid-holding recovery (14) during postirradiation incubation. It would be most difficult to detect this effect in these experiments. It is known that exponential-phase cells of *E. coli* B/r show liquid-holding recovery in non-nutrient medium after UV irradiation (Jagger, unpublished data). At 37°C, maximum recovery takes about 2 hr. To obtain a growth delay corresponding to a reciprocal of the growth-delay factor of 0.80 also takes approximately 2 hr for all wavelengths and represents about a 2.5-fold increase in cell number. Repair during growth would therefore be almost complete by the time of measurement at 50% transmittancy. However, if the reparable fraction is assumed to be the same for different wavelengths, it is not likely that the repair would greatly change the shape of the action spectrum. The same could be said about the influence of lethal sectoring, i.e., some of the surviving cells segregating into dividing and nondividing progeny (2). Furthermore, with the doses used, only a small fraction of the cells would undergo lethal sectoring, and there would be little effect on the action spectrum.

Damage to nucleic acids could lead to growth delay in several ways. It has been shown that DNA synthesis is the cellular function most sensitive to the action of far UV (3, 10), and it has been suggested that DNA is the primary site of action of UV for depressed RNA and protein synthesis (3). Some of these effects might be lethal. Hanawalt and Setlow (3) assumed that much of the DNA synthesized after recovery was biologically nonfunctional, as suggested by the high sensitivity of colony-forming ability to UV. In the present study, however, such a killing effect was eliminated in the measurement of growth delay by making a correction for killing. The growth delay we observe must, therefore, be due to sublethal damage. Such damage to DNA might delay replication of DNA, as well as transcription from DNA, and this could lead to a growth delay. On the other hand, there might be a more direct effect on protein synthesis, involving damage to ribosomal RNA (3), which could also be a chromophore.

We conclude that nucleic acid (either DNA or RNA) is probably the primary chromophore for induction of growth delay by wavelengths below

300 nm and that isoprenoid quinones are not important chromophores for growth delay in this region. Since radiation at 313 nm induces growth delay by a mechanism apparently different from that in the far UV (7), and probably involving quinones (6, 7), the crossover point between growth delay presumed to be due to damaged nucleic acid and growth delay presumed to be due to damaged quinones appears to lie in the region of 295 to 313 nm.

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