JOURNAL OF BACTERIOLOGY, Mar. 1974, p. 960-964
Copyright © 1974 American Society for Microbiology

Vol. 117, No. 3
Printed in U.S.A.

Lethal and Mutagenic Action of Black Light (325 to 400 nm) on *Haemophilus influenzae* in the Presence of Air

EMILIANO CABRERA-JUAREZ AND MERCEDES ESPINOSA-LARA
Departamento de Bioquímica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Carpio y Plan de Ayala, México 17, D.F., México

Received for publication 15 November 1973

Near-ultraviolet (UV) light (325 to 400 nm), in the presence of air and the absence of exogenous photosensitizing compounds, is lethal and mutagenic for *Haemophilus influenzae*. The lethal effect is the same for both wild type and streptomycin-resistant mutants, indicating that the mutants are not selected by the irradiation. The inactivation and mutagenicity show a large shoulder, suggesting the existence of repair systems. Filters were used to eliminate the possibility of short-UV irradiation. The effective radiation is between 325 to 400 nm. The lethal and mutagenic effects are higher during mid and late log phase than during early log or stationary phase.

The description of the lethal effect of near-ultraviolet (UV) light on bacteria was first reported in 1943 by Hollaender (7). Later, one of us reported that light from 330 to 380 nm, in the absence of exogenous photosensitizing compounds, produced the inactivation of transforming deoxyribonucleic acid (DNA) from *Haemophilus influenzae* (1). We showed that two processes were involved, one being oxygen dependent and another oxygen independent. After exhaustive purification of DNA, the oxygen-requiring inactivation no longer occurred. The oxygen-independent step which still occurred was called "black light inactivation." If the near-UV light was lethal to bacteria and inactivated DNA, we should expect it to be mutagenic. This report describes the lethal and mutagenic action of near-UV light on *H. influenzae* in the presence of air and the absence of exogenous photosensitizing compounds. A preliminary report of these findings has been published elsewhere (5).

MATERIALS AND METHODS

Microorganism. *H. influenzae* type Rd was used.

Culture of the cells to be illuminated. *H. influenzae* was grown in brain heart infusion (Difco) with or without agar; hemin and oxidized nicotinamide adenine dinucleotide (NAD^+) (final concentrations of 10 and 2 μg/ml, respectively) were used as supplements. The growth was followed in a Klett-Summerson photocolorimeter by using a red filter with a maximal transmittance at 660 nm. In these conditions, 1 optical density unit is equal to 2×10^{10} viable cells per ml. A 0.5-ml amount of competent cells (6) was placed in a 125-ml Erlenmeyer flask containing 25 ml of growth medium. The bacterial culture was incubated at 37 C with gentle shaking until an optical density (OD) of 0.3 was obtained.

Illumination medium. The fresh culture, at an OD of 0.3, was centrifuged to eliminate the complex medium, and the bacterial cells were then suspended at a concentration of 5×10^9 cells per ml in a solution containing 0.01 M sodium chloride, 0.01 M phosphate buffer, and 0.02% Tween 80 at pH 7.0 (2).

Illumination with near-UV light. The bacterial suspension was illuminated with a 15-W Westinghouse bulb (black light F15T8/BL) with a range of emission from 300 to 420 nm and a maximum at 350 nm. A 6-ml volume of the bacterial suspension was placed in a 9-cm petri dish, and a second petri dish containing 1.3% naphthalene solution 1 cm deep was placed on top of the dish containing the bacteria. The two petri dishes were gently shaken in an A. H. Thomas rotator and illuminated with the light source placed 5 cm above the bacterial suspension. The temperature of the cells was maintained at 3 C. A third petri dish containing 15% $KCr(SO_4)_2 \cdot 12H_2O$ (1 cm deep) was used occasionally over the petri dish containing the naphthalene solution; in this case, the distance of the lamp from the bacterial suspension was 8 cm. Samples of 0.5 ml were taken before and after different illumination times and kept at 3 C until all the samples were obtained.

Filters. The naphthalene and $KCr(SO_4)_2 \cdot 12H_2O$ solutions were used by the method of Kasha (8). The naphthalene filter (1.3%) was prepared with isooctane or ethanol as solvent. A 1-cm thick layer of this solution absorbed almost all the light below 325 nm, as measured in a Zeiss PMQII spectrophotometer (Fig. 1). The $KCr(SO_4)_2 \cdot 12H_2O$ filter was prepared by dissolving 15 g of $KCr(SO_4)_2 \cdot 12H_2O$ in distilled water and diluting it to 100 ml. This filter absorbs all the light from 380 to 450 nm (Fig. 1).

Determination of viable count and mutation frequency. To the 0.5 ml of the control or illuminated

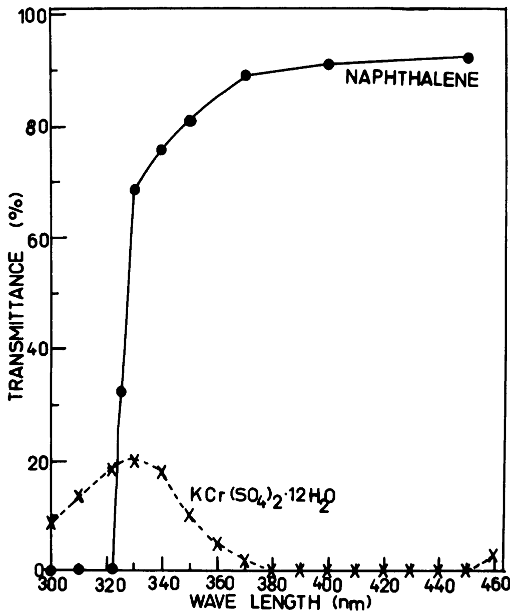

FIG. 1. *Transmission spectra of 1.3% naphthalene and 15% $KCr(SO_4)_2 \cdot 12H_2O$ solutions.*

samples, 1.5 ml of the growth medium was added and then the solution was incubated with gentle shaking at 37 C for 2 h. Appropriate dilutions were made in the growth medium without cofactors, and the viable count was determined by pour plating with the growth medium. Streptomycin-resistant mutants were measured by plating with 12 μg of streptomycin per ml in the growth medium. Mutants that could use protoporphyrin IX instead of hemin were determined when the organisms were plated in growth medium containing 1 μg of protoporphyrin IX per ml instead of hemin. The colonies were counted after incubation at 37 C for 24 to 48 h. The viability was expressed as a percentage of the control in the dark, and the mutation frequency was expressed the ratio of number of mutants to viable cells.

Streptomycin-resistant mutant of H. influenzae obtained by near-UV light. The wild-type strain was illuminated for 15 min. After segregation, the mutants were selected in the presence of 55 μg of streptomycin per ml. For isolation of a mutant, a stock culture was prepared and grown in the presence and absence of streptomycin. Mutants were resistant to at least 1 mg of the antibiotic per ml.

RESULTS

Action of black light on H. influenzae. The loss of viability after irradiation with unfiltered black light shows a large shoulder up to 45 min, followed by a rapid inactivation to 0.04% of survival at 105 min of illumination (Fig. 2). The effect on the mutation frequency followed a similar type of kinetics: at the beginning it increased slowly for 75 min and then very quickly. This behavior was the same for the two types of markers—resistance to streptomycin or utilization of protoporphyrin IX (Fig. 2).

Lethal and mutagenic effect of black light filtered through a naphthalene and $KCr(SO_4)_2 \cdot 12H_2O$ solutions on H. influenzae. The simultaneous introduction of a naphthalene filter and the $KCr(SO_4)_2 \cdot 12H_2O$ filter, that cut off light below 325 and above 380 nm (Fig. 1), did not change the general picture of the unfiltered black light. The loss of viability showed a large shoulder and then a rapid inactivation to 1.0% survival at 105 min of irradiation. The mutation frequency increased slowly for 75 min and then very quickly (Fig. 3). In the remaining experiments, the black light was filtered by naphthalene solution only.

Lethal action of light of 325 to 400 nm on wild-type and a streptomycin-resistant mutant of H. influenzae. Figure 4 shows that the lethal effect of the irradiation is the same for the wild type and a streptomycin-resistant mutant derived by mutagenesis with near-UV light, indicating that there is no selection against wild-type cells by these wavelengths and, thus, that the increase in the frequency of streptomycin-resistant cells obtained by irradiation of the wild-type strain is truly mutagenesis.

Lethal and mutagenic effect of light of 325 to 400 nm on H. influenzae at different stages of growth. It is of interest to note the effect of the irradiation on bacteria during different stages of growth. Samples of cells were taken at different points of the growth curve and irradiated. The early log-phase cells were taken when

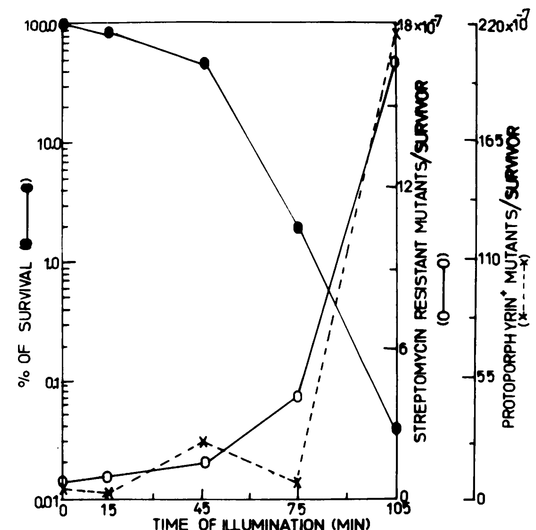

FIG. 2. *Lethal and mutagenic action of unfiltered black light on H. influenzae.*

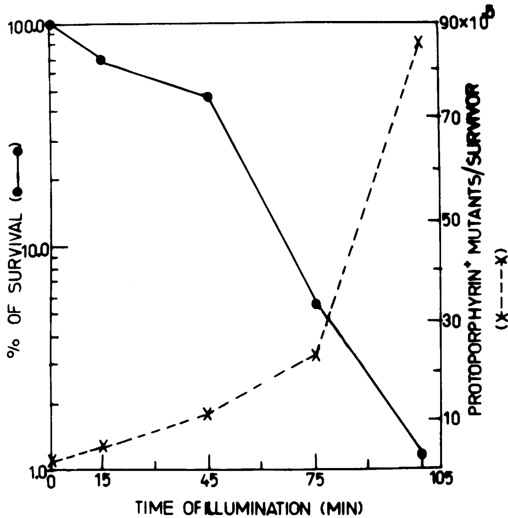

FIG. 3. *Lethal and mutagenic effect of black light, filtered through naphthalene and $KCr(SO_4)_2 \cdot 12H_2O$ solutions, on H. influenzae.*

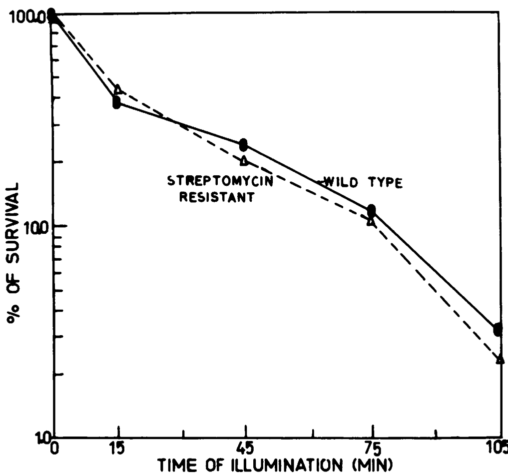

FIG. 4. *Lethal action of light of 325 to 400 nm on wild type and streptomycin-resistant mutants of H. influenzae.*

the OD was at 0.007 (A), distinct parts of log phase were at OD 0.05 (B), 0.20 (C), and 0.30 (D), and the beginning of the stationary phase was at OD 0.45 (E) (Fig. 5). The lethal effect was greater on the cells during log phase (B, C, D) than during early log (A) or stationary phase (E). In the first case, the curves appeared to have shoulders (Fig. 6). A small increase in the mutation frequency of the protoporphyrin-utilizing mutants was obtained by irradiation of cells at early log (A) or stationary phase (E).

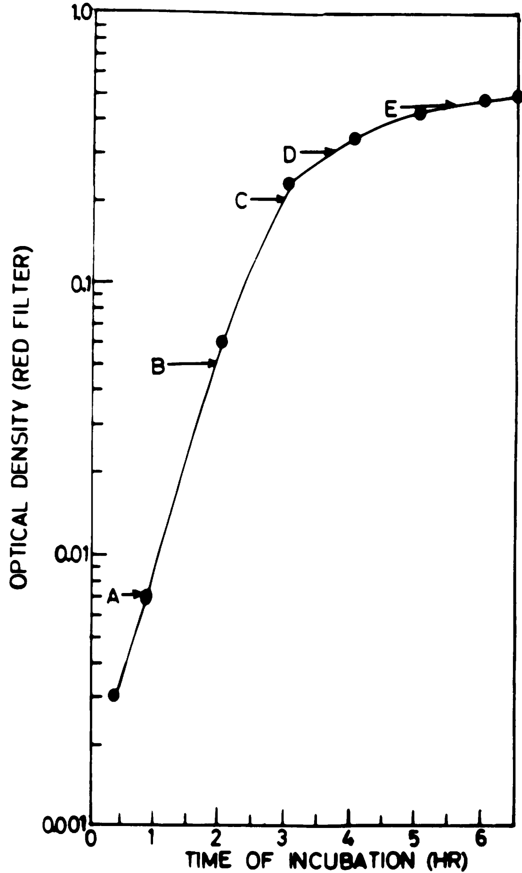

FIG. 5. *Growth curve of H. influenzae in brain heart infusion plus hemin and NAD^+ at 37 C. (A, B, C, D and E) Samples were taken at these stages of growth and illuminated; results are presented in Fig. 6 and 7.*

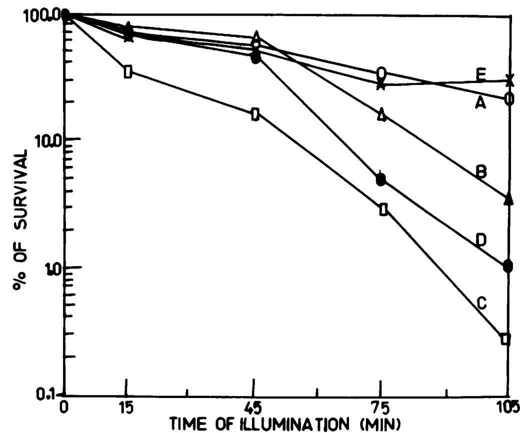

FIG. 6. *Lethal action of light of 325 to 400 nm on H. influenzae at different stages of growth. A, Early log phase; B, C, and D, log phase; E, stationary phase (see Fig. 5).*

Typical kinetics was observed with log-phase cells (B, C, D); first there is a small increase and after 45 or 75 min of irradiation the curve rises very quickly (Fig. 7). The frequency of mutation to streptomycin resistance exhibited similar kinetics (data not shown).

DISCUSSION

The unfiltered near-UV light (300 to 420 nm) in the presence of air and the absence of exogenous photosensitizing compounds showed lethal and mutagenic effects on *H. influenzae*. The viability was reduced to 0.04%, and the mutation frequency for the utilization of protoporphyrin or streptomycin resistance rose about 20 times with 105 min of irradiation. A similar near-UV-induced increase in mutation frequency of *E. coli* to T5 resistance has been described by Kubitschek (9) and Webb and Malina (11).

The near-UV light filtered simultaneously through naphthalene and $KCr(SO_4)_2 \cdot 12H_2O$ solutions produced a slight reduction of the lethal and mutagenic effects of the unfiltered light. These results are a consequence of the elimination of the light below 325 nm by the naphthalene filter and the partial absorption by the $KCr(SO_4)_2 \cdot 12H_2O$ filter of light between 330 and 380 nm. Under these conditions, we can say that the effective wavelengths were between 325 to 400 nm. A similar effect on the oxygen-independent inactivation of transforming DNA

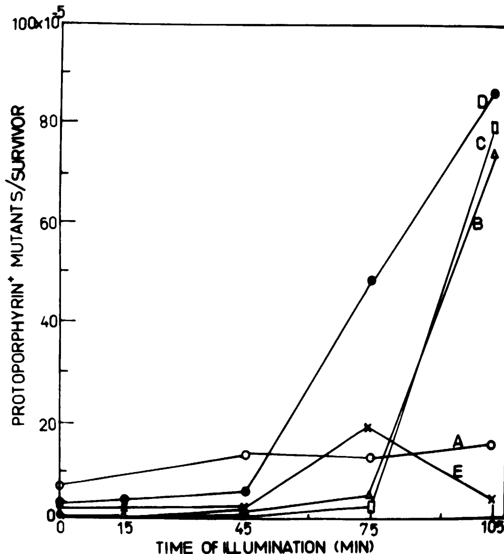

FIG. 7. *Mutagenic effect of light of 325 to 400 nm on H. influenzae at different stages of growth. A, Early log phase; B, C, and D, log phase; E, stationary phase (see Fig. 5).*

of *H. influenzae* by near-UV light has been described (1). In *E. coli* the effective wavelength was found to be around 365 nm (9), and beyond 340 nm on *Salmonella typhimurium* (3).

The action of 325- to 400-nm irradiation is dependent on the growth stage of *H. influenzae;* on the other hand, there is little or no change in UV sensitivity as a function of growth stage (10). The lethal and mutagenic effect of 325- to 400-nm illumination is greatest during the log phase of growth, except for very early log phase. If we assume that the DNA in the log phase has larger number of small, single-stranded regions than the stationary phase, then we would expect the effect of near-UV on *H. influenzae* to be higher in the log phase since its denatured DNA is more sensitive to this irradiation (1). These results agree with the findings of Eisenstark (3) for *Salmonella typhimurium*, where the lethal effect of near-UV light is greatest when the cells are dividing. This interpretation does not explain our results with the early-log-phase cells because they also are dividing cells. The simultaneous increase of the two types of mutants by irradiation of *H. influenzae* at the log phase is in agreement with the results obtained in *E. coli* (12). Our results with stationary-phase cells of *H. influenzae* are different from those obtained by Webb and Tai (12) with *E. coli*. We obtained a small increase of the two types of mutants; Webb and Tai obtained only one type of mutant. We do not know whether this is a real difference or we need to look for different types of mutants during the stationary phase.

The lethal and mutagenic effect of irradiation of 325 to 400 nm in the presence of air and absence of photosensitizing compounds on *H. influenzae* shows a large shoulder. This suggests the existence of a repair system or possibly that the light has two different mechanisms of action. Results with transforming DNA (1) and our initial findings on *H. influenzae* support the possibility of two mechanisms in response to irradiation, one that requires oxygen and the other that does not. In *E. coli*, these two different actions of the near-UV light have been described (9, 11). Preliminary results in our laboratory support the hypothesis of a dark-repair system in *H. influenzae* that repairs the damage produced by the 325- to 400-nm irradiation. There is evidence in *Salmonella typhimurium* also for the existence of a repair system for the damage produced by near-UV light (4).

ACKNOWLEDGMENTS

We thank A. Eisenstark for his comments and careful review of the manuscript and R. Maldonado and R. Alcántara for useful discussions.

E.C.-J. was supported by a fellowship from the Sección de Especiálización Docente e Investigación Cientifica y Tecnológica, subordinate of the Comisión de Operación y Fomento de Actividades Académicas of the Instituto Politécnico Nacional.

LITERATURE CITED

1. Cabrera-Juárez, E. 1964. "Black light" inactivation of transforming deoxyribonucleic acid from *Haemophilus influenzae*. J. Bacteriol. **87**:771-778.
2. Cabrera-Juárez, E., and R. M. Herriott. 1963. Ultraviolet irradiation of native and denatured transforming deoxyribonucleic acid from *Haemophilus influenzae*. J. Bacteriol. **85**:671-675.
3. Eisenstark, A. 1970. Sensitivity of *Salmonella typhimurium* recombinationless (rec) mutants to visible and near-visible light. Mutat. Res. **10**:1-6.
4. Eisenstark, A., and D. Ruff. 1970. Repair in phage and bacteria inactivated by light from fluorescent and photolamps. Biochem. Biophys. Res. Commun. **38**:244-248.
5. Espinosa-Lara, M., and E. Cabrera-Juárez. 1972. Efecto de la luz cercana al visible (300-400 nm) sobre *Haemophilus influenzae*. Rev. Lat.-Amer. Microbiol. **14**:103-106.
6. Goodgal, S. H., and R. M. Herriott. 1961. Studies on transformation of *Haemophilus influenzae*. I. Competence. J. Gen. Physiol. **44**:1201-1227.
7. Hollaender, A. 1943. Effect of long ultraviolet and short visible radiation (3500-4900 Å) on *E. coli*. J. Bacteriol. **46**:531-541.
8. Kasha, M. 1948. Transmission filters for the UV. J. Opt. Soc. Amer. **38**:929-934.
9. Kubitschek, H. E. 1967. Mutagenesis by near visible light. Science **155**:1545-1546.
10. Modak, S. P., and J. K. Setlow. 1969. Synthesis of deoxyribonucleic acid after ultraviolet irradiation of sensitive and resistant *Haemophilus influenzae*. J. Bacteriol. **98**:1195-1198.
11. Webb, R. B., and M. M. Malina. 1967. Mutagenesis in *Escherichia coli* by visible light. Science **156**:1104-1105.
12. Webb, S. J., and C. C. Tai. 1969. Physiological and genetic implications of selective mutation by light at 320-400 nm. Nature (London) **224**:1123-1124.