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"BLACK LIGHT" INACTIVATION OF TRANSFORMING DEOXYRIBONUCLEIC ACID FROM *HAEMOPHILUS INFLUENZAE*

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ABSTRACT

CABRERA-JUÁREZ, EMILIANO (Instituto Politecnico Nacional, Mexico, D.F., Mexico). "Black light" inactivation of transforming deoxyribonucleic acid from *Haemophilus influenzae*. J. Bacteriol. **87**:771-778. 1964.—The biological activity (intrinsic genetic markers or nitrous acid mutable regions) of transforming deoxyribonucleic acid (DNA) from *Haemophilus influenzae* has been inactivated by "black light" (BL) by two mechanisms: (i) photodynamic action (oxygen-dependent) and (ii) "BL inactivation" (oxygen-independent). The BL inactivation is greater in denatured than in native DNA, and it is dependent on the pH. It does not depend on the temperature, and the damage produced is stable. The effective wavelength of inactivation is between 330 and 360 mμ. The BL inactivation is not reactivated by photoreactivating enzyme or nitrous acid. The BL and ultraviolet inactivations are additive, suggesting that the changes produced by BL and ultraviolet irradiation on transforming DNA are different. T_2 phage was also inactivated by BL. The nature of the photochemical changes produced in DNA by BL is not known.

The relative absorption of 320- to 400-mμ radiation by deoxyribonucleic acid (DNA) is so low that it is often considered to be negligible. The inactivation of transforming DNA by such radiation (black light, BL) would, therefore, not be expected unless photodynamic substances were present (Clare, 1956; Bellin and Oster, 1960). Since photodynamic inactivation requires oxygen (Clare, 1956; Simon and Van Vunakis, 1962), the effect of displacement of the oxygen by nitrogen prior to irradiation should permit a decisive answer on this possibility.

The present report shows that, even under nitrogen, purified transforming DNA from *Haemophilus* is inactivated slowly by radiations of wavelengths between 320 and 380 mμ.

Denatured DNA was found to be much more sensitive than native DNA, and at pH 4.8 the radiation was more destructive than at neutrality.

MATERIALS AND METHODS

Microorganism. H. influenzae type "d" was used.

General methodology. The preparation of DNA and competent cells, and most of the laboratory techniques, were as described by Goodgal and Herriott (1961). Some samples of DNA were prepared first by this method, and then isopropanol was used to precipitate DNA and separate it from ribonucleic acid (RNA), oligoribonucleotides, and polysaccharides, as Marmur (1961) recommended.

DNA. Native DNA was extracted from *H. influenzae* resistant to 25 μg/ml of novobiocin (Cathomycin) and is referred to as C_{25}DNA. In a comparable way, DNA was obtained from cell strains resistant to 250 μg/ml of streptomycin Sm_{250}DNA and to 20 μg/ml of erythromycin (E_{20}DNA). Denatured DNA was prepared by heating native DNA at 100 C for 5 min and quenching in ice water; the DNA concentration during this treatment was 40 μg/ml. After experimental treatment of denatured DNA, it was renatured by heating for 1 hr at pH 7 and 66 C in the following manner. In general, a 10- or 1-ml sample of 2 μg/ml of denatured DNA dissolved in 0.3 M sodium chloride-0.01 M sodium citrate was placed in a tube and incubated in a 6-liter bath of water maintained at 65 to 67 C for 1 hr, after which the system was allowed to cool to 30 C. The cooling took about 2 hr.

BL illumination of denatured or native transforming DNA. The apparatus used to illuminate with BL is the same as that used in the experiments on photoreactivation (Rupert, 1960). It consists of a bank of three closely spaced, parallel, BL fluorescent tubes (General Electric F20 T12 BL, 20-w; range of emission, 300 to 450 mμ with a maximum at 350 mμ) placed approxi-

mately 3 cm below a glass-bottomed water bath adjusted to 37 C. Screw-capped test tubes (15 by 125 mm) containing the DNA samples rested upright on the glass bottom directly over the center fluorescent tube. Absorption filters were made by introducing 60 ml of certain solutions into a 9-cm petri dish. The solution was 1 cm deep. The petri dishes were then placed on the glass bottom of the bath, and the tubes with DNA were suspended just above the petri dish. No water was in the bath during use of the filter. The temperature varied from 25 to 30 C.

To adjust the samples to pH 4.8 prior to illumination with BL, 0.5 ml of acetate buffer was added to 0.5 ml of denatured or native DNA solution in 0.03 M sodium chloride-0.01 M sodium citrate. The final concentration of buffer was 0.05 M and of the DNA was 20 μg/ml; the pH of the mixture was 4.8. Some samples were treated with BL, and duplicates were kept in the dark as controls. At intervals, samples were neutralized to pH 7.4 by a tenfold dilution into 0.02 M Na_2HPO_4 and 0.3 M sodium chloride. The samples of denatured DNA were then renatured. The samples of renatured and native DNA were diluted, and their transforming activity was assayed. The control (100% transforming activity) consisted of 0.5 ml of denatured or native DNA mixed with 0.5 ml of acetate buffer plus 9.0 ml of 0.02 M Na_2HPO_4 and 0.3 M saline. This control was put through the renaturation process and then assayed. The transforming activity of the BL-illuminated or dark-incubated samples was calculated as the percentage of this control. In the experiments of Tables 1, 2, 3, and 4 and Fig. 4, the control consisted of a DNA sample treated with acetate buffer and incubated in the dark during the same time as the sample treated with BL.

When the samples were illuminated with BL at pH 7, phosphate buffer was used instead of acetate buffer. The final concentration of buffer was 0.01 M; the pH of the DNA-buffer mixture was 7. After illumination, the samples were diluted tenfold in 0.01 M sodium citrate-0.3 M sodium chloride. The control consisted of a sample with buffer incubated in the dark for a period equal to the maximal time that any experimental sample was illuminated, then diluted, renatured, and titered. The titer was not different from a duplicate control kept in the refrigerator. The transforming activity of the BL-illuminated samples was calculated as the percentage of this control.

When the BL illumination was studied in an atmosphere of nitrogen, the DNA-buffer mixture was introduced into a tube (20 by 170 mm), and nitrogen was bubbled through it for 5 min; then the tube was tightly closed and exposed to the BL. A tube with methylene blue showed that under the above conditions a little reoxidation was observed only after 5 hr. Controls were run in the dark.

Filters. The naphthalene and $KCr(SO_4)_2 \cdot 12H_2O$ solutions were used as suggested by Kasha (1948). The naphthalene filter (1.3%) was prepared with isooctane as solvent. A 1-cm light path through this solution absorbed almost all the light below 330 mμ as measured in a Beckman DU spectrophotometer. The $KCr(SO_4)_2$ filter was prepared by dissolving 15 g of $KCr(SO_4)_2 \cdot 12H_2O$ in distilled water and diluting to 100 ml. This filter transmits light between 230 and 400 mμ. The reduced nicotinamide adenine dinucleotide (NADH) filter (Siegel, Montgomery, and Bock, 1959) was prepared in distilled water. This solution strongly absorbs between 300 and 380 mμ. All the filter solutions were freshly prepared.

Ultraviolet irradiation of denatured transforming $C_{25}DNA$. The details of this method were described previously (Cabrera-Juárez and Herriott, 1963). The concentration of DNA was 5 μg/ml in 0.3 M saline-0.005 acetate buffer-0.02 M Na_2HPO_4.

Treatment with nitrous acid or buffer. To 1 volume of a mixture of acetate buffer and 2 M $NaNO_2$ in 0.15 M sodium chloride or of acetate buffer in 0.15 M sodium chloride was added 1 volume of BL-inactivated denatured DNA. The final concentrations were: sodium nitrite, 1 M; buffer, 0.05 M; and DNA, 20 μg/ml. The initial pH was 4.8. The reaction mixture was incubated at 37 C for 30 min, at which time the samples were neutralized to pH 7 by a tenfold dilution with 0.02 M Na_2HPO_4 in 0.3 M sodium chloride; then the samples were renatured.

Photoreactivation. The photoreactivating enzyme from baker's yeast (YPRE; Rupert, 1960) was applied to BL-inactivated denatured C_{25}-DNA. Inactivation by BL at pH 4.8 was permitted until 10% of the transforming activity remained. The solution was then neutralized to pH 7, renatured, and dialyzed to remove the nitrite (Cabrera-Juárez, 1963). To 0.5 ml of this

dialyzed DNA at 2 μg/ml was added 0.5 ml of a heated or unheated 1:10 dilution in citrate-saline of a stock YPRE [freshly prepared by dissolving 100 mg of ammonium sulfate-fractionated yeast extract (Rupert, 1960) in 10.0 ml of 1% glycerine in citrate saline], and the mixtures were incubated as described by Rupert (1960).

Transformation procedure. The assay procedure described by Goodgal and Herriott (1961) was followed, but during the uptake of C_{25}DNA or Sm_{250}DNA a simple synthetic solution was used (Cabrera-Juárez and Herriott, 1963) instead of "Elev" broth, and the overlaying method was followed. In the studies of the uptake of E_{20}DNA, Brain Heart Infusion (Difco) and the pour plate procedure were used. The titer or number of transformed cells in the reaction mixture was arrived at by multiplying the observed number of transformants by the dilution factor. The comparable titer or transforming activity of the different samples was calculated as percentage of the control.

New transforming markers. To look for new transforming markers (Horn and Herriott, 1962) in DNA after treatment with BL and nitrous acid or with BL or nitrous acid alone, the following assay was used. The reaction mixture consisted of 3 ml of Difco Brain Heart medium [supplemented with hemin and nicotinamide adenine dinucleotide (NAD)], 2×10^8 cells per ml, and 0.2 μg/ml of DNA. This mixture was shaken 150 min at 37 C, and then the number of streptomycin (5 μg/ml)-resistant mutants and viomycin (150 μg/ml)-resistant mutants was determined as described before (Cabrera-Juárez and Herriott, 1963).

Phage methodology. Escherichia coli B and T_2 phage were used. The general methods described by Adams (1959) were followed.

RESULTS

Preliminary experiments showed that native or denatured transforming DNA was inactivated by illumination with BL. This inactivation was greater at pH 4.8 than at neutrality, and the denatured DNA was about ten times more sensitive to BL illumination than was native DNA. The C_{25} marker was more sensitive to BL illumination than were E_{20} or Sm_{250} markers.

To determine whether the differences in BL sensitivity were due to the markers or to some other factors in the DNA preparations, the markers were mixed and purified again by ethanol precipitation and then retested for sensitivity to BL. The results showed that the sensitivity of the C_{25} marker to the BL illumination was less after precipitation, whereas the other two markers remained unchanged. This suggests that the difference in BL sensitivity might be due to a higher concentration of some photodynamic compound in the C_{25}DNA than in the other two types of DNA. To test this possibility, denatured C_{25}DNA was BL-illuminated in air and in nitrogen. DNA was inactivated less in nitrogen than in air (Table 1), supporting the notion that photodynamic action was probably responsible for some inactivation of the C_{25}DNA preparation.

TABLE 1. *Black light inactivation of denatured C_{25}DNA at pH 4.8 under air or nitrogen*

DNA*	Time of black light illumination	Per cent transforming activity	
		Air	Nitrogen
	min		
A	0	100	100
	60	6	24
B	0	100	100
	60	41	47

* A = DNA prepared by the method of Goodgal and Herriott (1961); B = DNA prepared by a combination of the methods of Goodgal and Herriott (1961) and Marmur (1961).

Elimination of photodynamic factors. To eliminate any photodynamic complications, it was necessary to prepare DNA which showed the same inactivation under nitrogen or air. Repeated isopropanol precipitation of the DNA, as recommended by Marmur (1961), yielded DNA satisfying this criterion (see Table 1). When this DNA was mixed with Sm_{250}DNA prepared by the same methods, then denatured and illuminated with BL, the inactivation (Table 2) of C_{25} marker was again the same in air or nitrogen, and it was identical to the inactivation obtained when the C_{25}DNA was illuminated alone (Table 1). This was also true for the Sm_{250} marker. In all the succeeding experiments, these preparations of DNA which showed the same degree of inactivation in air or nitrogen were used. In subsequent experiments, illumination in an air atmosphere was used for simplicity.

Effective inactivating wavelength. The manufacturer indicates that the BL tubes have a range of emission between 300 and 450 mμ with a

TABLE 2. *Black light inactivation of a mixture of denatured $C_{25}DNA$ and $Sm_{250}DNA$ at pH 4.8 under air or nitrogen**

Marker	Time of black light illumination	Per cent transforming activity	
		Air	Nitrogen
	min		
C_{25}	0	100	100
	60	50	46
Sm_{250}	0	100	100
	60	41	36

* DNA was prepared by a combination of the methods of Goodgal and Herriott (1961) and Marmur (1961).

TABLE 3. *Effect of NADH filter and time of illumination at pH 4.8 on black light inactivation of denatured $C_{25}DNA$**

Filter	Transmittance	Time	Per cent transforming activity	
			Dark	Black light
	%	*min*		
Water	100	60	100	29
		120	100	—
NADH (0.05 mM)	50	60		48
		120		21

* DNA was prepared by a combination of the methods of Goodgal and Herriott (1961) and Marmur (1961).

maximum at 350 mμ. The introduction of a naphthalene solution which absorbs all wavelengths below 330 mμ had no effect on the inactivation of DNA. This shows that stray or small doses of ultraviolet light, below 330 mμ, are not responsible for the inactivation. In the same way, solutions of $KCr(SO_4)_2$ (Kasha, 1948) eliminated radiation above 400 mμ. The inactivating light must lie between 330 and 400 mμ. A NADH filter, which absorbs light between 300 and 380 mμ, inhibits the inactivation. The fact that passage of the light through a NADH filter, which reduced the transmittancy 50%, necessitated doubling the time of illumination to equal the inactivation of the control DNA (Table 3) suggests that the regions of absorption of BL by DNA and NADH are very similar.

Effect of pH on BL inactivation of denatured and native DNA. The rate of inactivation by BL was the same for denatured C_{25}DNA and Sm_{250}-DNA, and the course of the reaction is represented by one-hit curves (Fig. 1). A comparison of the time of illumination necessary to reduce the transforming activity to 70% at pH 4.8 and 7 shows that the denatured DNA is about 20 times more sensitive to BL at pH 4.8 than at pH 7. Similar results (Fig. 2) were obtained during the illumination of native DNA. A comparison of the results in Fig. 1 and 2 shows that at pH 4.8 the denatured C_{25}DNA is five times as sensitive to BL as is native DNA. Preliminary observations showed that renatured DNA has about the same sensitivity as does native DNA.

Temperature and stability of BL damage. The BL inactivation was not affected by the temperature of the sample between 25 and 80 C. The BL damage was stable; a sample analyzed for transforming activity 1 month after BL inactivation was the same as that obtained immediately after BL treatment.

Destruction of nitrous acid mutable regions in denatured DNA by BL inactivation. BL, besides inactivating intrinsic markers of transforming DNA, was also capable of destroying the capacity of this DNA to form new markers with nitrous acid. Table 4 contains the number of strepto-

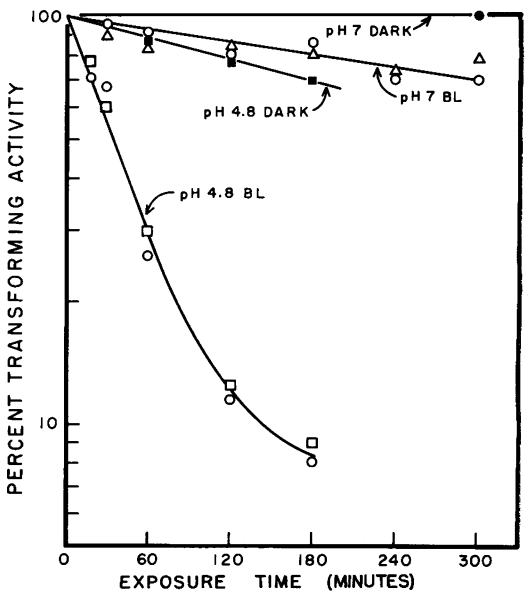

FIG. 1. *Black light (BL) inactivation of a mixture of denatured C_{25} and Sm_{250} DNA. Symbols: ● = C_{25} and Sm_{250} DNA held in the dark at pH 7; ○ and △ = C_{25} and Sm_{250} DNA exposed to BL at pH 7; ■ = C_{25} and Sm_{250} DNA held in the dark at pH 4.8; □ and ○ = C_{25} and Sm_{250} DNA exposed to BL at pH 4.8.*

mycin-resistant or viomycin-resistant transformants produced by DNA treated with nitrous acid acting on denatured C_{25}DNA which had been exposed to varying doses of BL and then renatured. It is clear that the number of nitrous acid-induced genetic markers conferring resistance to 5 μg/ml of streptomycin or 150 μg/ml of viomycin began to be reduced at 30 min of illumination; the markers disappeared after 120 min of BL illumination. Illumination alone of control DNA failed to produce heritable changes in the DNA which conferred even marginal levels of resistance to either of these antibiotics.

Attempts to reactivate the BL-damaged DNA. It is known that the ultraviolet damage to transforming DNA can be reactivated by use of the photoreactivating enzyme (Rupert, 1960) or nitrous acid (Carbrera-Juárez and Herriott, 1963; Cabrera-Juárez, 1963). It was of interest to apply these two procedures to BL-inactivated denatured C_{25}DNA to obtain reactivation. Samples of denatured DNA were treated with different doses of BL; then they were treated with the same doses of nitrous acid or buffer and renatured. The nitrous acid did not reactivate the BL damage to DNA (Fig. 3). On the contrary, nitrous acid inactivated the DNA even more. It appears that the illumination sensitized the denatured DNA to the nitrous acid inactivation. Nitrous acid destroyed about half of the activity prior to illumination (Fig. 3). In drawing the "expected" curve, it was assumed that after BL illumination nitrous acid reduced the residual activity by this same fraction.

A sample of denatured C_{25}DNA inactivated with BL until 10% survival was not reactivated by YPRE. A control sample with heated YPRE did not show any change either. Denatured C_{25}DNA inactivated with ultraviolet light at pH 4.8 or 7 until 10% survival *was* photoreactivated with the same enzyme preparation.

Inactivation of denatured C_{25}DNA with BL and ultraviolet irradiation. To determine whether BL sensitizes denatured DNA to ultraviolet irradiation, a sample of denatured DNA was illuminated with BL until about 15% survived. This sample and a control (pretreated with buffer) were subjected to different doses of ultraviolet light, and then renatured and tested for transforming activity. The rate of ultraviolet inactivation of both samples was the same (Fig. 4).

Failure to observe some physical-chemical changes

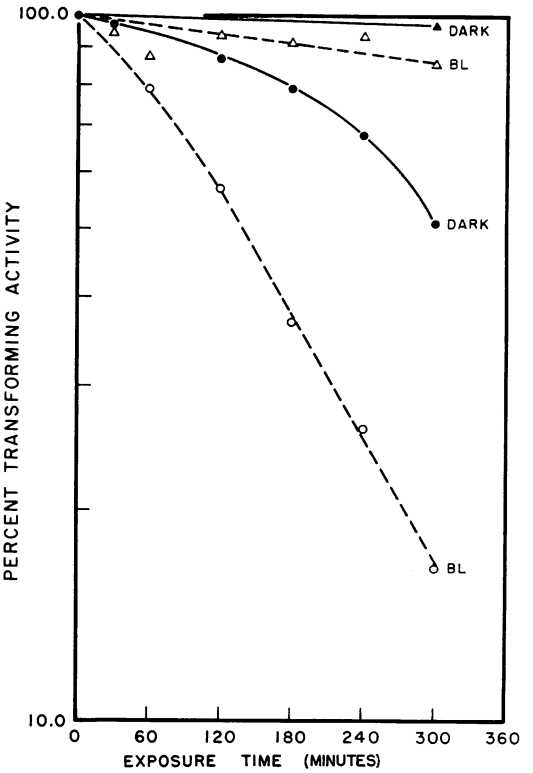

FIG. 2. *Black light inactivation of native C_{25} DNA. Circles, pH 4.8; triangles, pH 7. Isopropanol was used in the last stage of DNA purification.*

TABLE 4. *Effect of black light (BL) inactivation of denatured C_{25}DNA on the formation of new markers with HNO_2**

Time of BL illumination (min)	New markers†	
	Streptomycin (5 μg/ml)-resistant mutants per ml of mixture	Viomycin (50 μg/ml)-resistant mutants per ml of mixture
Control (no BL, no HNO_2)	5.6×10^4	4.1×10^4
0	3.1×10^5	2.7×10^5
30	1.2×10^5	9.0×10^4
60	9.5×10^4	6.2×10^4
120	5.8×10^4	3.8×10^4
180	5.5×10^4	3.7×10^4

* DNA was prepared by a combination of the methods of Goodgal and Herriott (1961) and Marmur (1961). DNA concentration, 40 μg/ml; pH, 4.8. Nitrous acid treatment: DNA concentration, 20 μg/ml; see Materials and Methods.

† See Materials and Methods.

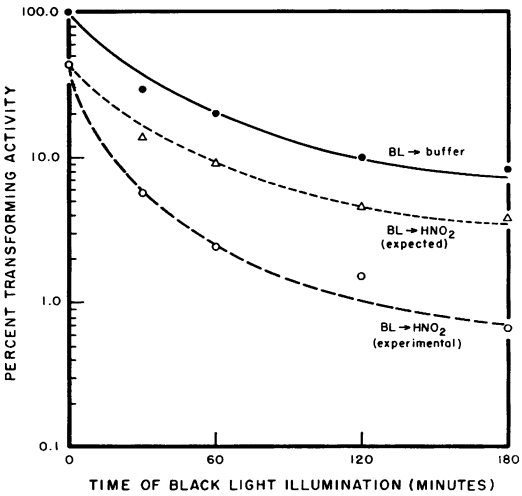

FIG. 3. *Nitrous acid on black light-inactivated denatured $C_{25}DNA$. Isopropanol precipitation was the final step in purification of this DNA. For BL illumination: DNA concentration, 40 μg/ml; pH 4.8. For nitrous acid or buffer treatment: DNA concentration, 20 μg/ml.*

after BL illumination. It was of some interest to learn whether the BL inactivation of transforming activity was accompanied by some physical-chemical changes. We were unable to detect changes in the absorption spectrum of denatured or native transforming DNA after BL illumination, or any effect of this illumination on the hyperchromicity produced in transforming DNA by heat denaturation in the presence of formaldehyde. BL also produced no changes in the absorption spectra of deoxycytidylic, deoxyguanylic, deoxyadenylic, and thymidylic acid after 10 hr of BL illumination at pH 4.8 or 7.

BL illumination of T_2 phage. The BL inactivation is not a specific feature of transforming DNA. The plaque-forming ability of T_2 phage suspended in phosphate buffer (pH 7) was inactivated also (Fig. 5). At pH 4.8, the inactivation of the control was very high, but it was possible to detect a faster inactivation of the phage by BL at this pH.

DISCUSSION

The photodynamic inactivation of transforming principle (Bellin and Oster, 1960) and the photodynamic action on DNA (Simon and Van Vunakis, 1962) are dye-sensitized and oxygen-requiring phenomena. The present studies indicate that transforming DNA from *H. influenzae* isolated by the method of Goodgal and Herriott (1961) is inactivated by BL in absence of any added dye by two mechanisms: one requiring the presence of oxygen (possibly photodynamic inactivation) and the other not requiring oxygen (nonphotodynamic action). When the transforming DNA was purified by a combination of the methods of Goodgal and Herriott (1961) and Marmur (1961), the BL inactivation was by the nonphotodynamic mechanism only. The present paper is concerned with this nonphotodynamic inactivation of transforming DNA.

The BL inactivation of transforming principle is greater in denatured than in native DNA, and it is more effective at an acidic pH than at neutral pH. Its action is not dependent on the temperature. The effects of this radiation appear to be stable and include the destruction of nitrous acid-mutable regions.

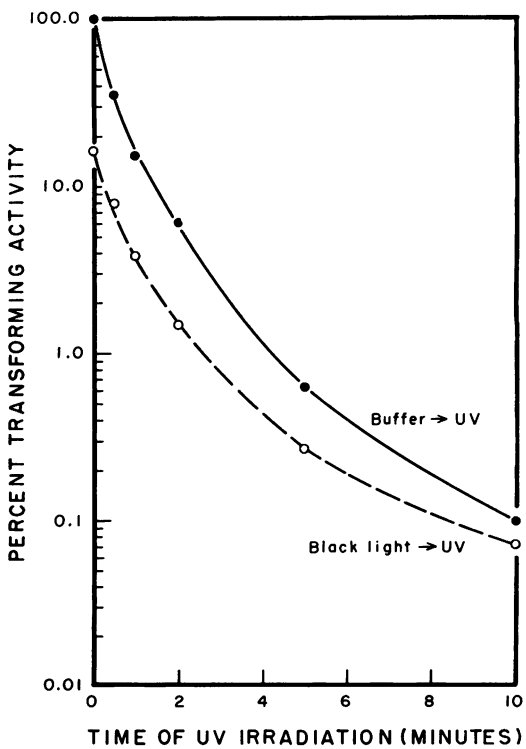

FIG. 4. *Ultraviolet irradiation of black light-inactivated denatured $C_{25}DNA$. Isopropanol-precipitated DNA was used in this experiment. For BL illumination: DNA concentration, 50 μg/ml; pH 4.8; time, 3 hr. For ultraviolet irradiation: DNA concentration, 5 μg/ml; pH 7.*

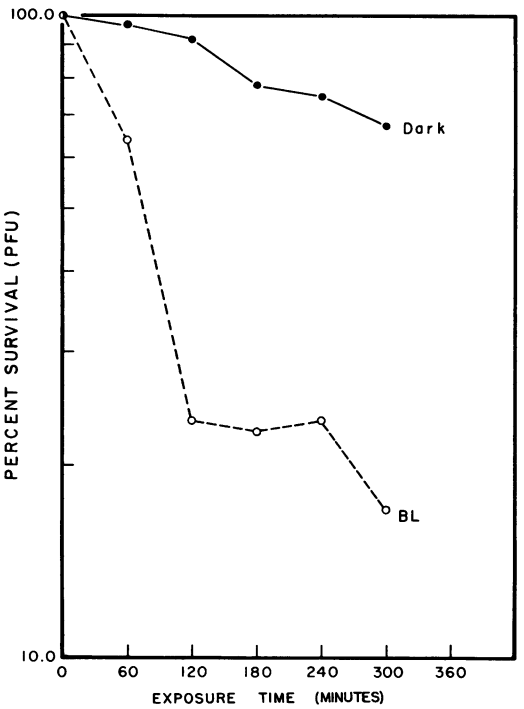

FIG. 5. *Black light illumination of T_2 phage. Phage concentration during illumination: 10^8 plaque-forming units/ml in 0.01 M phosphate buffer; pH 7; temperature, 37 C.*

The effective inactivating light is in the range of 330 to 360 mμ. Zamenhof et al. (1956) and Bellin and Oster (1960) did not find inactivation of transforming DNA by wavelengths above 400 mμ. Wacker, Mennigmann, and Szybalski (1962), in studies on the effect of "visible light" on 5-bromouracil-labeled DNA, indicate that the effective wavelength is between 313 and 366 mμ.

The sensitivity to ultraviolet irradiation is the same in denatured and native DNA, and it does not change between pH 5 and 8 (Cabrera-Juárez and Herriott, 1963). These properties which are so different from the response to BL, together with the failure to reactivate the BL damage with YPRE or nitrous acid and the finding that transforming DNA pretreated with BL did not change its rate of inactivation with ultraviolet treatment, show that the action of ultraviolet light and BL on DNA are different. No change in absorption spectra of the DNA has been seen after BL inactivation.

The photoreactivation of transforming DNA after ultraviolet irradiation is made at pH 7 and involves the action of BL on native DNA plus the yeast enzyme (Rupert, 1960). In this case, the BL inactivation and photodynamic action on DNA are small, but when denatured DNA is used or the DNA has not been sufficiently purified these effects could be complicating factors reducing the photoreactivation process. A control with heated YPRE illuminated with BL will indicate the extent of this complication.

The plaque-forming capacity of T_2 phage was inactivated by BL, as was noted earlier by Stahl et al. (1961). The finding by these authors that T_2 bacteriophage containing 5-bromodeoxyuridine was more sensitive to illumination is evidence that the DNA is the site of action of BL.

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