# ULTRAVIOLET INACTIVATION AND PHOTOPRODUCTS OF TRANSFORMING DNA IRRADIATED AT LOW TEMPERATURES

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ABSTRACT Solutions of *Haemophilus influenzae* transforming DNA were irradiated at temperatures ranging from 25°C to −196°C. Temperature dependence of the formation of thymine-containing dimers was closely correlated with inactivation of transforming activity; in general, both dimerization and inactivation decreased with decreasing temperature. The fraction of nonphotoreactivable damage increased with increasing dose at low temperatures. The nonphotoreactivable spore-type photoproduct was formed at low temperatures with a maximum at −100°C, a temperature at which the nonphotoreactivable biological inactivation was also a maximum. Intrastrand cross-linking, like dimer formation, decreased with decreasing irradiation temperature.

## INTRODUCTION

Ultraviolet (UV)-induced cyclobutane pyrimidine dimers are mainly responsible for the UV inactivation of *Haemophilus influenzae* transforming DNA at 25°C (Setlow and Setlow, 1962), a temperature at which dimers are readily formed in DNA (Setlow and Carrier, 1966). However, the yield of such dimers is relatively low at −196°C (Rahn, 1966), probably because the majority of the bases are in a conformation unfavorable for dimerization (Rahn and Hosszu, 1968). Lerman and Tolmach (1959) have shown that the inactivation of pneumococcus transforming DNA is several times smaller at −196°C than at 25°C. We have found a close correlation between inactivation and dimers in *H. influenzae* transforming DNA irradiated at temperatures ranging from −196°C to 25°C.

At low temperatures, an additional photoproduct is formed in DNA—a nonphotoreactivable, thymine-containing photoproduct that is chromatographically indistinguishable from a photoproduct obtained from UV-irradiated bacterial spores (Donnellan and Setlow, 1965; Donnellan and Stafford, 1968; Smith and Yoshikawa, 1966). After maximum photoreactivation of UV-irradiated DNA with yeast photoreactivating enzyme, the thymine-containing cyclobutane dimers are no longer present (Setlow and Boling, 1963) and the remaining biological inactivation must be due to photoproducts which are not affected by photoreactivation. Therefore, we

compared the temperature dependence of formation of the spore photoproduct in *H. influenzae* DNA with the nonphotoreactivable damage to the transforming activity. Similar experiments correlating the temperature dependence of the spore photoproduct with biological activity have already been reported for the spores themselves (Donnellan et al., 1968).

## METHODS

### *DNA*

*Haemophilus influenzae* DNA was prepared by the method of Marmur (1961). For radioactive labeling of the DNA, the bacteria were grown in 3.5% brain-heart infusion (Difco Laboratories, Detroit, Mich.), supplemented with 5 μg/ml hemin, 5 μg/ml L-histidine, 2 μg/ml nicotinamide adenine dinucleotide phosphate, and tritiated thymidine. The resulting DNA had a specific radioactivity of about 6000 cpm/μg. For irradiation, the DNA (1–40 μg/ml in M/15 phosphate buffer, pH 7) was diluted 1:1 with ethylene glycol, which forms a glass at temperatures below −80°C and is effectively transparent to the UV wavelengths at all the temperatures used. The transforming ability of the DNA was not affected by the ethylene glycol. Following irradiation, the DNA was diluted 300-fold for the transformation assay. Transformation methods were as previously described (Goodgal and Herriott, 1961). Two markers were used: resistance to streptomycin (250 μg/ml) and to cathomycin (2.5 μg/ml). The streptomycin marker is considerably more sensitive to UV than cathomycin marker.

### *UV Irradiation and Photoreactivation*

Long wavelengths were obtained by passing the output from a 500-watt high-pressure mercury lamp through a thin-film interference filter (15 nm bandpass) having a transmittance peak at 280 nm. For short-wavelength irradiation a Bausch and Lomb Incorporated (Rochester, N. Y.) monochromator was used in conjunction with the same type of lamp. A GE 15-watt low pressure mercury lamp provided the 254 nm radiation. Intensity measurements were made with a thermistor bolometer. The DNA samples were irradiated at temperatures between −196° and 25°C in 3-mm I.D. quartz tubes placed in a quartz vacuum-jacketed tube through which precooled gaseous nitrogen was passed. A Varian variable-temperature accessory was used to obtain the desired temperatures. Photoreactivation of the DNA was carried out, as previously described (Setlow et al., 1965), under blacklight illumination at 37°C for times up to 1½ hr, which was more than sufficient time to achieve maximum reactivation of transforming ability in the case of all the UV doses used. The photoreactivating enzyme was purified about 800-fold according to Muhammed (1966).

### *Photoproduct Measurement*

*Labeled Photoproducts.* After irradiation the labeled DNA was hydrolyzed with 98% formic acid for 30 min at 175°C. The resulting hydrolysates were chromatographed on Whatman No. 1 paper with *n*-butanol:acetic acid:water (80:12:30). The radioactivity, contained in strips cut from the chromatograms, was then eluted with water and measured in a liquid scintillation counter with a dioxane-naphthalene scintillator.

### *Cross-Links*

Reversible OD changes following heat denaturation of DNA were taken as the measure of cross-links. DNA samples in the buffer-glycol mixture were irradiated in quartz tubes at

various temperatures and then subjected to 90°C for 2 min, followed by quick cooling. The absorbance was recorded before and after heating in order to estimate the extent of Type I reversibility (Geiduscheck, 1961).

## RESULTS AND DISCUSSION

Fig. 1 *a* shows the dose dependence of UV inactivation at several temperatures before and after maximum photoreactivation. The data are presented on a square root plot, which yields a straight line at lower UV doses (Rupert and Goodgal, 1960). The UV sensitivity without photoreactivation, when the pyrimidine dimers are present, decreases with decreasing temperatures of irradiation; it is considerably greater at 25°C than at −180°C, in agreement with Lerman and Tolmach's results (1959) with pneumococcus transforming DNA. On the other hand, the residual inactivation after maximum photoreactivation is greatest in the case of irradiation at −99°C, demonstrating that more nondimer damage is produced at this temperature than at 25°C. Nevertheless, most of the inactivation at −99°C is due to pyrimidine dimer formation, since photoreactivation leads to a large recovery of activity.

Fig. 1 *b* shows the amount of thymine-containing dimer and spore-type photo-

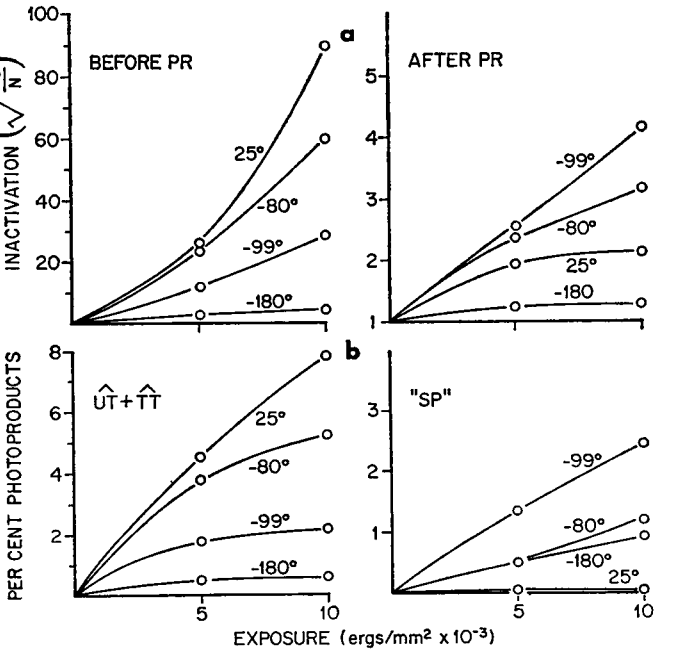


FIGURE 1 (*a*) Inactivation at 254 nm of *H. influenzae* transforming DNA (streptomycin marker) at different centrigrade temperatures (given as ° on curves), before and after maximum photoreactivation (PR). $N_0$ and $N$ represent the number of transformations obtained from unirradiated and irradiated DNA, respectively. (*b*) Photoproducts in the DNA of Fig. 1 a. The photoproduct $\widehat{UT}$ is obtained from $\widehat{CT}$ upon hydrolysis.

TABLE I

PHOTOREACTIVABILITY OF TRANSFORMING DNA AND THE RATIO OF PHOTOPRODUCTS FORMED AT −196°C

| Dose (at 254 nm) | "sp"/($\widehat{TT}$ + $\widehat{UT}$) | Photoreactivable sector |
|---|---|---|
| *ergs/mm²* | | |
| 5,000 | 0.92 | 0.90 |
| 15,000 | 1.2 | 0.87 |
| 45,000 | 2.8 | — |
| 135,000 | 4.1 | 0.21 |

product formed in the same irradiated DNA used for the Fig. 1 *a* data. The spore-type photoproduct, sp, unlike the dimer, is unaffected by the photoreactivating enzyme. At −180° and −99°C the yield of pyrimidine dimers changes very little with dose after the initial 5000 ergs/mm (Setlow and Carrier, 1966). However, sp continues to form as the dose is increased, and is formed most readily at −99°. These data suggest that sp contributes to nonphotoreactivable inactivation of transforming DNA at this temperature.

The low-temperature data of Fig. 1 *b* show that the ratio of dimers to sp decreases with increasing dose. If sp is a biological lesion at lower irradiation temperatures, we would then expect the photoreactivable fraction of biological damage to decrease with increasing dose at such temperatures. In Table I the ratio of sp to dimers is shown to increase by a factor of about 4.5 from the lowest to the highest dose, whereas the photoreactivable sector (Setlow, 1963) decreased by a similar factor, going from 90% to 20%. In contrast to these results, the photoreactivable sector for DNA irradiated at room temperature at 265 nm appears to *increase* at very large doses (Setlow, 1963).

Fig. 2 shows the thymine-containing dimers and the biological inactivation of DNA (cathomycin marker) irradiated with a single dose at a series of temperatures. It is seen that except for the point at 25°C there is a close correspondence between the temperature variation of dimer formation and the inactivation, providing further evidence that pyrimidine dimers make a large contribution to the biological inactivation of DNA, even at low temperatures. Similar results were obtained with the streptomycin marker.

In Fig. 3, the amount of sp and the residual inactivation after photoreactivation is plotted versus temperature for a single dose. Both of these quantities reach a maximum at ∼−100°C. However, it is clear that this photoproduct cannot account for all the nonphotoreactivable biological inactivation over the temperature range studied, since (*a*) at 25°C there is negligible sp but considerable inactivation after photoreactivation, and (*b*) at −180°C the opposite situation exists, with a significant amount of sp present but little inactivation after photoreactivation. Thus, at the

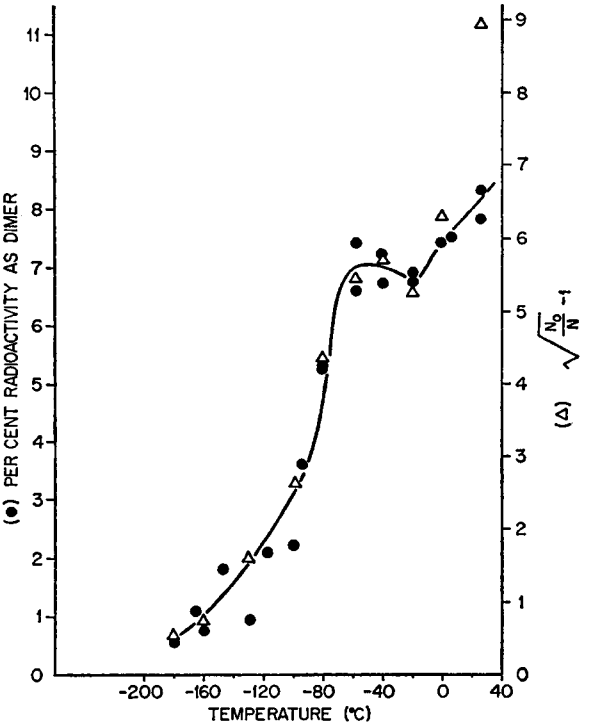


FIGURE 2 Inactivation at 254 nm of *H. influenzae* transforming DNA (cathomycin marker) and dimer formation in $^3$H-labeled *H. influenzae* DNA by a dose of $10^4$ ergs/mm$^2$ at different temperatures. $N_0$ and $N$ are the number of transformations resulting from the unirradiated and irradiated DNA, respectively. The parameter used as a measure of the biological inactivation, $\sqrt{N_0/N} - 1$, represents the slope of the UV dose curve at $10^4$ ergs/mm$^2$.

lowest and highest temperatures we have investigated, the amount of nonphotoreactivable biological damage is not closely correlated with the amount of sp.

The formation of another photoproduct, the cross-link, was measured as a function of temperature (Fig. 4). This photoproduct forms with increasing difficulty as the temperature is lowered. Hence we rule out the possibility that the enhancement of nonphotoreactivable damage at −99°C is due to cross-link formation, and we conclude that sp, which is most readily formed at this temperature, contributes significantly to the biological damage.

The spore photoproduct differs from the dimer in that it does not show the phenomenon of short-wavelength reversibility (Setlow and Setlow, 1962; Rahn and Hosszu, 1968). Use of this phenomenon was previously made to demonstrate that pyrimidine dimers contribute to the biological inactivation of transforming DNA at room temperature (Setlow and Setlow, 1962); i.e., a large dose of long-wavelength radiation was followed by short-wavelength irradiation, which split most of the

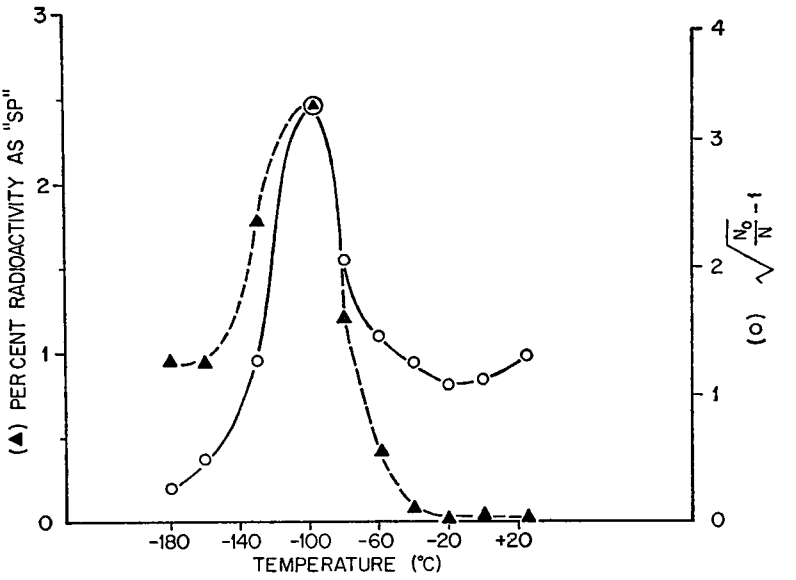


FIGURE 3 Comparison of "sp" formation at 254 nm following a dose of $10^4$ ergs/mm² at different temperatures with the residual inactivation of *H. influenzae* transforming DNA (streptomycin marker) after maximum photoreactivation.

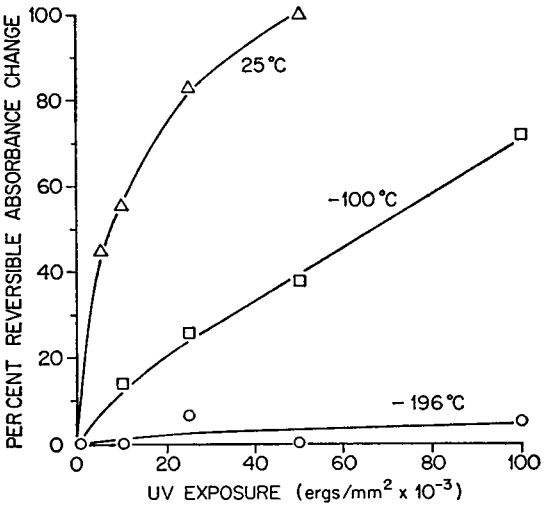


FIGURE 4 Variation in the degree of cross-linking with irradiation temperature, as indicated by the per cent reversible absorbance change at 260 nm, of *H. influenzae* DNA following heat denaturation and quick cooling. DNA was irradiated at 254 nm for various doses at the temperatures shown, and the absorbance was measured before and after heat denaturation.

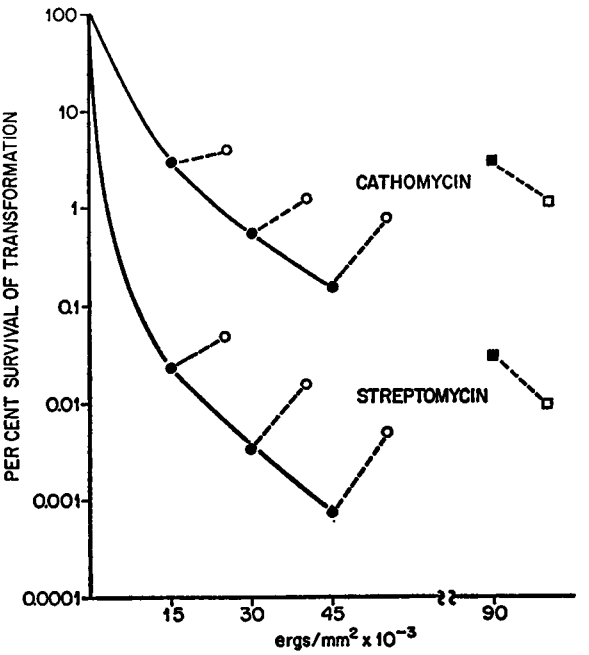


FIGURE 5 Long-wavelength UV inactivation of two markers of *H. influenzae* transforming DNA at either −196°C or 25°C followed by short-wavelength irradiation at 25°C to reverse the dimer damage. ●, 280 nm (25°C); ○, 280 nm (25°C) + 238 nm; ■, 280 nm (−196°C); □, 280 nm (−196°C) + 238 nm. The samples irradiated at −196°C were intermittently thawed and refrozen in order to obtain a level of inactivation comparable with that obtained at 25°C.

dimers and caused the transforming activity to increase (Setlow and Setlow, 1962). We have repeated this experiment, as shown in Fig. 5, although here the long-wavelength irradiation (280 nm) was either at 25°C or at −196°C. After the low temperature irradiation, done at high doses to achieve a level of inactivation comparable to that obtained at 25°C, the DNA was thawed and further irradiated at 238 nm. This short-wavelength irradiation caused a decrease in survival rather than an increase, which is observed when the 280 nm irradiation is at 25°C. This experiment again shows that some of the biological damage at low temperature is different from that at room temperature. The lack of short-wavelength reversibility of this damage is consistent with the notion that sp is partly responsible for the low-temperature inactivation of transforming DNA.

The relative effectiveness of sp relative to $\widehat{TT}$ and $\widehat{CT}$ can be estimated in the following way, if we assume that sp represents all of the nonphotoreactivable damage. At −99°C after 5000 ergs/mm², about 80% of the inactivation is photoreactivable (Fig. 1 *a*); it is presumed to be due to $\widehat{CT}$ + $\widehat{TT}$, which amounted to 1.8% of the thymine (Fig. 1 *b*). The nonphotoreactivable 20%, then, is assumed to be due to the

spore-type photoproduct which amounted to 1.4% of the thymine. On the basis of thymine content, the spore-type photoproduct is judged to be only about 20% as effective as $\acute{T}\grave{T}$ and $\widehat{CT}$ in inactivating the DNA. Stafford and Donnellan (1968) have reported that the pyrimidine dimers are 11 times more effective in killing vegetative cells than spore photoproducts are in killing spores. The relative biological effectiveness of different photoproducts may reflect differences in the ability of the organism to repair them.

## SUMMARY

The UV inactivation of transforming activity at low doses for all temperatures studied is strongly correlated with the production of photoreactivable damage, i.e., the production of thymine-containing dimers, the yield of which decreases with decreasing temperature. The nonphotoreactivable damage resulting from irradiation at temperatures in the region of $-100°C$ is to a large extent accounted for by the spore-type photoproduct, which is estimated to be less than 20% as lethal as a thymine-containing dimer.

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