ULTRAVIOLET IRRADIATION OF NATIVE AND DENATURED TRANSFORMING DEOXYRIBONUCLEIC ACID FROM *HAEMOPHILUS INFLUENZAE*

EMILIANO CABRERA-JUÁREZ[1] AND ROGER M. HERRIOTT

Department of Biochemistry, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland

Received for publication 29 October 1962

ABSTRACT

CABRERA-JUAREZ, EMILIANO (Johns Hopkins University School of Hygiene and Public Health, Baltimore, Md.) AND ROGER M. HERRIOTT. Ultraviolet irradiation of native and denatured transforming deoxyribonucleic acid from *Haemophilus influenzae*. J. Bacteriol. **85**:671–675. 1963.—Two antibiotic-resistance markers in denatured deoxyribonucleic acid (DNA) from *Haemophilus influenzae* showed about the same sensitivity to ultraviolet irradiation as in native DNA. The resistance of markers in denatured DNA to ultraviolet (UV) light increases with alkalinity up to pH 12. New transforming markers or genetic "lethals" were not produced by in vitro irradiation of either native or denatured DNA. The UV irradiation of the denatured DNA reduces its capacity to form new markers with nitrous acid. Nitrous acid reactivated some of the intrinsic marker destroyed by UV irradiation of denatured transforming DNA.

The exposure of bacteria to ultraviolet (UV) irradiation has in many instances led to the formation of mutants (Zelle, 1955; Witkin, 1956). Virus-infected cells often release mutant viruses if they have been irradiated during infection (Weigle, 1953; Weigle and Dulbecco, 1953; Jacob, 1954). It is a little surprising, then, that the cases of mutants arising after direct irradiation of extracellular viruses or transforming deoxyribonucleic acid (DNA) are very limited (Krieg, 1959; Nester and Lederberg, 1961). In view of a recent observation (Horn and Herriott, 1962) that single-stranded (denatured) *Haemophilus* DNA was very sensitive to the mutagenic action of nitrous acid whereas the native form was not, it was decided to irradiate denatured DNA with UV and after renaturation to look for new transformable markers in the DNA. It was reasoned that perhaps the positive mutagenic action on cells occurs when, for a brief time, some of the DNA may be in single-strand form. However, despite many analyses for a variety of markers, no new markers (mutants) were detected after irradiation of either denatured or native hemophilus transforming DNA.

[1] On leave from: Departmento de Biofísica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México, D.F., México. Supported by the John Simon Guggenheim Memorial Foundation.

MATERIALS AND METHODS

General methodology. The preparation of DNA from *Haemophilus influenzae*, competent cells media, and most of the laboratory techniques have been described previously (Goodgal and Herriott, 1961).

DNA. We used native or denatured C_{25} DNA [DNA extracted from *H. influenzae* resistant to 25 μg/ml of cathomycin (first isolated by Mary J. Voll)]. In some experiments we tested native and denatured Sm_{250} DNA (DNA extracted from *H. influenzae* resistant to 250 μg/ml of streptomycin). [This marker is Smα or Sm^{2000} of Hsu and Herriott (1961) and was first isolated by Alexander and Leidy (1953), but only 250 μg/ml of streptomycin was used in the present experiments for screening.] Denatured DNA was obtained by heating and quenching according to Marmur and Lane (1960). After treatment with UV light, nitrous acid, or both, the denatured DNA was renatured by the method of Marmur (1960), which involves heating for 1 hr at pH 7 and 66 C with the following steps. In general, a 1-ml sample of 20 μg/ml of denatured DNA, dissolved in 0.5 M NaCl-0.01 M sodium citrate, was placed in a tube and incubated in a bath of 6 liters of water at 65 to 67 C for 1 hr. Then the tube was transferred to 1 liter of water at 65 to

67 C, and it was allowed to cool to 30 C, which took about 2 hr.

UV irradiation of native or denatured transforming DNA. For UV irradiation in general, DNA at a concentration of 20 μg/ml in 0.15 M NaCl-0.01 M sodium citrate was exposed for various time periods at 45 cm from a 15-w General Electric germicidal lamp with an output of approximately 25 ergs per mm^2 per sec. The solutions irradiated in a petri dish were approximately 1 mm thick and were mixed by rotation of the dish during irradiation.

Treatment with nitrous acid. The method of Horn and Herriott (1962) was followed. To 1 volume of a mixture (acetate buffer and 2 M $NaNO_2$ in 0.15 M saline, pH 4.8) was added 1 volume of denatured DNA. The final concentration of buffer was 0.05 M, and the DNA concentration was 50 μg/ml. It was incubated at 37 C for 30 min, at which time the samples were neutralized to pH 7.4 by a tenfold dilution with 0.02 M Na_2HPO_4 in 0.3 M sodium chloride. The concentration of DNA was now 5 μg/ml and was annealed as described above.

Transformation procedure. The assay procedure described by Goodgal and Herriott (1961) was followed, but during the uptake of DNA a solution containing 0.1 M sodium chloride, 0.01 M phosphate buffer, and 0.02% Tween 80 (pH 7.0) was used instead of "Elev" broth. The titer in the reaction mixture was calculated, and from this the percentage of residual transforming activity of the different samples relative to the control.

New transforming or lethal markers in UV-irradiated DNA. To look for new transforming or lethal markers in the UV-irradiated DNA, the following method was used. The reaction mixture consisted of 3.0 ml of Difco Brain Heart Infusion [supplemented with hemin and diphosphopyridine nucleotide (DPN)], 2 × 10^8 cells/ml, and 0.1 μg/ml of DNA. This is equivalent to about 20 molecules of DNA per cell. This mixture was shaken for 150 min at 37 C. The cells were then washed and diluted with Eugonbroth (BBL), and plated on the following different media: (a) Brain Heart Infusion Agar plus hemin plus DPN, (b) medium a plus 5 μg/ml of streptomycin, (c) medium a plus 5 μg/ml of erythromycin, (d) Brain Heart Infusion Agar plus DPN plus 5 μg/ml of protoporphyrin, (e) Brain Heart Infusion Agar plus hemin. After 24 to 48 hr of incubation at 37 C, the colonies were counted. The results in medium a yielded the viable count of the cells in the reaction mixture. If the sample of DNA has some lethal markers, there should be a significant difference between the samples with and without DNA. The results in media b, c, d, or e give, respectively, the number of mutants resistant to 5 μg/ml of streptomycin, to 5 μg/ml of erythromycin, and those mutants capable of utilizing 5 μg/ml of protoporphyrin and also DPN-independent mutants. If the sample of DNA contains some new marker(s) produced by irradiation and of the type screened for in this work and if this new marker is integrated by the competent cells, there should be an increase in the number of resistant cells relative to the control without DNA.

RESULTS

Inactivation of genetic markers in denatured DNA by irradiation. The sensitivity of genetic markers in denatured (single-stranded) transforming DNA to ultraviolet irradiation has not previously been described because there was no way of evaluating the effect until Marmur and Lane (1960) described the renaturation procedure. As a preliminary to the problem of

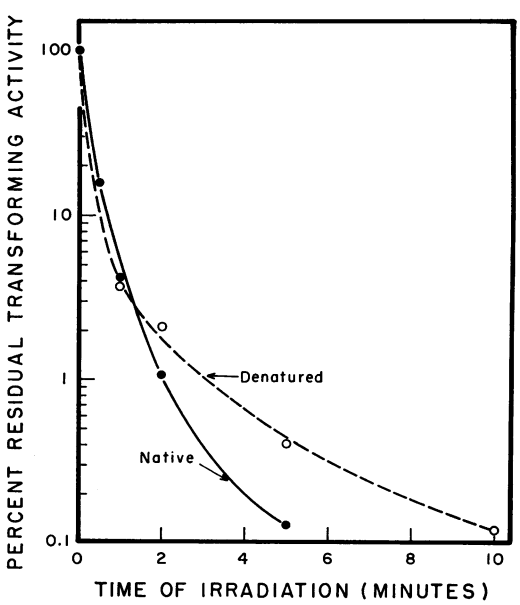

FIG. 1. *Ultraviolet inactivation of cathomycin (25 μg/ml) resistance marker in native and denatured DNA.*

generating new markers by irradiation, an examination of the relative sensitivity to UV of two different markers in native and heat-denatured *Haemophilus* DNA was determined.

The experimental results in Fig. 1 and 2 show that there is little difference in sensitivity of the native and denatured forms of transforming DNA. The slightly greater resistance seen for denatured C_{25} DNA in Fig. 1 is reversed when one views the action on the Sm^{250} marker shown in Fig. 2.

Effect of pH on ultraviolet inactivation of genetic markers in denatured DNA. Curves of the surviving transforming activity after irradiation of denatured *Haemophilus* C_{25} DNA at different pH values, followed by renaturation at pH 7 before testing, are shown in Fig. 3. It seems clear from these figures that the C_{25} marker in denatured DNA is considerably more stable to irradiation at pH 12 than in the more neutral solutions.

Attempts to produce genetic changes in DNA by in vitro irradiation. Nitrous acid had been so successful in generating new markers in he-

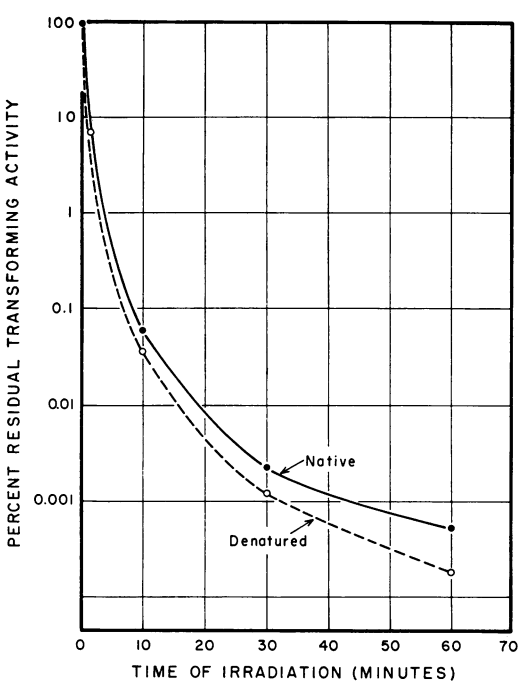

FIG. 2. *Ultraviolet inactivation of streptomycin (250 μg/ml) resistance marker in native and denatured DNA.*

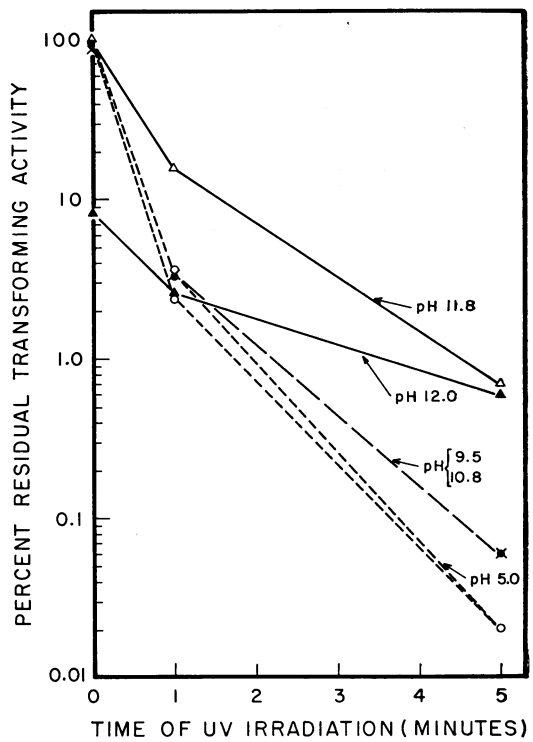

FIG. 3. *Effect of pH on the ultraviolet inactivation of cathomycin (25 μg/ml) resistance marker in denatured DNA.*

mophilus DNA that we were encouraged to try UV light under similar circumstances. Markers similar to those generated by nitrous acid were examined. However, using separately native, as well as denatured, DNA and irradiating with varying doses and at different pH values failed to produce heritable changes in the DNA leading to resistances in the cells to marginal levels of any of the antibiotics. Nor did the irradiated DNA contain genetic "lethals," for saturating levels of the irradiated DNA failed to prevent any detectable fraction of the cells from growing.

Destruction of nitrous acid mutable regions in denatured DNA by UV irradiation. When it was observed that UV irradiation failed to produce new genetic units in denatured DNA, it was of interest to learn whether this treatment had destroyed the capacity to form new markers with nitrous acid. Column I of Table 1 contains the number of streptomycin-resistant transformants produced with C_{25} DNA which had been dena-

tured, exposed to varying doses of UV irradiation, then exposed to a constant treatment with nitrous acid, and finally renatured. It is amply clear that the number of nitrous acid-induced S^5 genetic markers was not reduced by 20 sec of irradiation, but after 100 sec only one-fourth of the number remained.

The data in column II show that when these two treatments were reversed (i.e., the nitrous acid treatment was followed by the UV irradiation) the nitrous acid-induced markers began to fall at a lower radiation dosage. After only 5 sec of exposure, there was a drop of 50% in the new markers. At first glance, this seems to suggest that the nitrous acid-induced markers are more sensitive to UV light than the corresponding regions in untreated nucleic acid (column I), but the experiments described below suggest a different explanation; namely, that nitrous acid reverses the UV damage to DNA and in so doing allows it to yield new markers with nitrous acid.

The initial or intrinsic C_{25} genetic marker in denatured C_{25} DNA was exposed to UV light for varying periods. Samples were removed for test, and the remainder of each sample was given a constant exposure to nitrous acid. All samples were renatured and tested for intrinsic C_{25}-transforming activity. The results are plotted in Fig. 4. The interesting point is that apparently the nitrous acid partially reversed the destruction inflicted by UV irradiation. This did not occur in irradiated native DNA. Simple chemical reversal of irradiation damage to genetic materials has not previously been reported, although photoenzymatic reversal has (Rupert, Goodgal, and Herriott, 1958).

TABLE 1. *Effect of ultraviolet (UV) irradiation and nitrous acid on denatured C_{25} DNA*

Time of UV irradiation	Streptomycin (5 μg/ml) resistant mutants per ml of mixture	
	Column I*	Column II†
	$UV \rightarrow HNO_2$	$HNO_2 \rightarrow UV$
sec		
Control‡	2.30×10^4	2.30×10^4
0	1.46×10^5	1.46×10^5
5	1.50×10^5	6.94×10^4
20	1.48×10^5	5.27×10^4
100	4.13×10^4	2.18×10^4
300	2.75×10^4	2.15×10^4
600	2.75×10^4	1.95×10^4

* Effect of UV irradiation on the formation of new markers with HNO_2.

† Sensitivity of the new markers produced by nitrous acid to UV irradiation.

‡ No irradiation and no nitrous acid treatment.

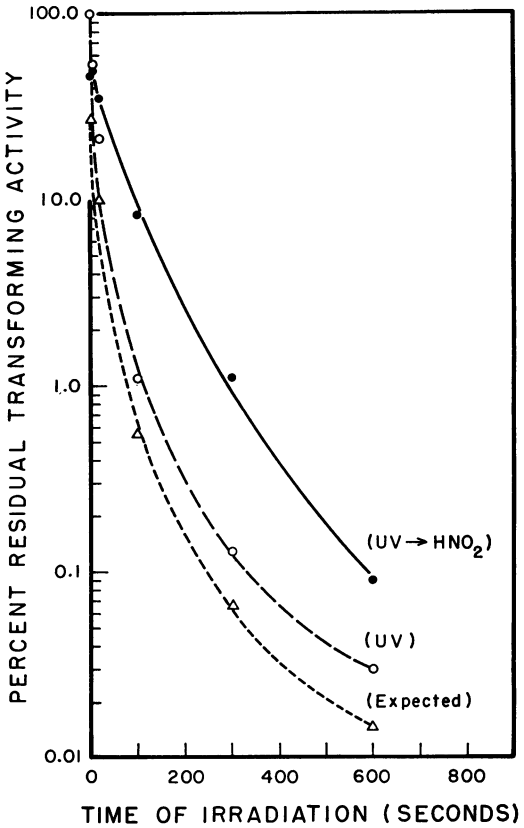

FIG. 4. *Effect of nitrous acid on ultraviolet-inactivated cathomycin (25 μg/ml) resistance marker in denatured DNA.*

DISCUSSION

Our inability to observe new markers in irradiated native or irradiated denatured DNA followed by renaturation is not readily explained. Experiments using P^{32}-labeled DNA indicate that both are taken up by competent cells. If the irradiation forms a preponderance of damaged markers, some of these might well have found their way into the genome of the host cell and resulted in the death of the cell, i.e., "lethals." However, none was detected, so we must conclude that somehow UV light produces changes which prevent its genetic integration and expres-

sion in cells. It is not likely that interstrand linkage produced by irradiation can account for the failure to observe new genetic effects, especially in the case of denatured DNA. Furthermore, if the irradiation were performed at about 50 to 60 C, it would be possible to decide whether intrastrand linkage played a significant role.

The auxotrophic mutants which Nester and Lederberg (1961) produced with UV irradiation of *Bacillus subtilis* DNA were of such a low frequency that the techniques used in the present work might not have detected them.

If further work confirms these observations that nitrous acid reversal of UV damage is restricted to denatured DNA, it will be the second case of the development of a genetic property via denatured DNA. It was reported earlier (Horn and Herriott, 1962) that new genetic markers developed during nitrous acid treatment of denatured DNA and not with native DNA. Since both these cases involved nitrous acid, it may be that comparable changes developed in the native DNA but cross-linking of the DNA strands by nitrous acid (Geidushek, 1961) prevented their biological expression.

ACKNOWLEDGMENTS

This investigation was supported by a U.S. Public Health Service training grant, 2G-73, from the Division of General Medical Sciences, research grant E-1218 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service; and Atomic Energy Commission contract AT(30-1)-1371.

LITERATURE CITED

ALEXANDER, H. E., AND G. LEIDY. 1953. Induction of streptomycin resistance in sensitive *Hemophilus influenzae* by extracts containing deoxyribonucleic acid from resistant *Hemophilus influenzae*. J. Exptl. Med. **97**:17–31.

GEIDUSCHEK, P. 1961. "Reversible" DNA. Proc. Natl. Acad. Sci. U.S. **47**:950–955.

GOODGAL, S. H., AND R. M. HERRIOTT. 1961. Studies on transformation of *Hemophilus influenzae*. I. Competence. J. Gen. Physiol. **44**:1201–1227.

HORN, E. E., AND R. M. HERRIOTT. 1962. The mutagenic action of nitrous acid on "single-stranded" (denatured) hemophilus transforming DNA. Proc. Natl. Acad. Sci. U.S. **48**:1409–1416.

HSU, Y. C., AND R. M. HERRIOTT. 1961. Studies on transformations of *Hemophilus influenzae*. III. The genotypes and phenotypic patterns of three streptomycin-resistant mutants. J. Gen. Physiol. **45**:197–204.

JACOB, F. 1954. Mutation d'un bactériophage induite par l'irradiation des seules bactéries-hôtes avant l'infection. Compt. Rend. **238**: 732–734.

KRIEG, D. R. 1959. Induced reversion of T4r$_{II}$ mutants by ultraviolet irradiation of extracellular phage. Virology **9**:215–227.

MARMUR, J., AND D. LANE. 1960. Strand separation and specific recombination in deoxyribonucleic acids: biological studies. Proc. Natl. Acad. Sci. U.S. **46**:453–461.

NESTER, E. W., AND J. LEDERBERG. 1961. Linkage of genetic units of Bacillus subtilis in DNA transformation. Proc. Natl. Acad. Sci. U.S. **47**:52–55.

RUPERT, C. S., S. H. GOODGAL, AND R. M. HERRIOTT. 1958. Photoreactivation in vitro of ultraviolet inactivated *Hemophilus influenzae* transforming factor. J. Gen. Physiol. **41**:451–471.

WEIGLE, J. J. 1953. Induction of mutations in a bacterial virus. Proc. Natl. Acad. Sci. U.S. **39**:628–636.

WEIGLE, J. J., AND R. DULBECCO. 1953. Induction of mutations in bacteriophage T3 by ultraviolet light. Experientia **19**:372–373.

WITKIN, E. M. 1956. Time, temperature and protein synthesis: a study of ultraviolet-induced mutation in bacteria. Cold Spring Harbor Symp. Quant. Biol. **21**:123–140.

ZELLE, M. R. 1955. Effects of radiation on bacteria, p. 365–430. *In* A. Hollaender [ed.], Radiation biology, vol. 2. McGraw-Hill Book Co., Inc., New York.