Nitrous Acid Reactivation of Ultraviolet-Irradiated Transforming DNA from *Hemophilus influenzae*

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ABSTRACT Partial recovery of ultraviolet-damaged denatured or native transforming DNA from *Hemophilus influenzae*, has been obtained by exposing the irradiated DNA in the denatured form to nitrous acid. Some factors that affect this recovery are described. An erythromycin marker (E_{20}) was not reactivated. The UV damage reactivable by nitrous acid is different from that repaired by the photoreactivating enzyme from bakers' yeast. The pretreatment with nitrous acid affords a slight protection for denatured C_{25} DNA and Sm_{250} DNA against ultraviolet irradiation, but this pretreatment sensitized the E_{20} DNA to this irradiation.

In vitro photoenzymatic reversal of the action of ultraviolet irradiation on DNA[1] has been studied for several years (1–3) but restoration by simple chemical means has not been described. While determining whether ultraviolet irradiation of denatured *Hemophilus* DNA destroyed its capacity to form new genetic markers with nitrous acid it was observed (4) that nitrous acid restored some of the intrinsic transforming activity lost through irradiation.

The present communication confirms and extends these observations on the reactivating property of nitrous acid for ultraviolet-irradiated transforming DNA. The action is restricted to denatured DNA and the site of reactivation appears to be different from that restored by the photoenzyme from yeast.

MATERIALS AND METHODS

Microorganism, *Hemophilus influenzae* type "d".

General Methodology The preparation of DNA, competent cells, and media and most of the laboratory techniques have been described previously (5).

[1] Abbreviations: DNA, desoxyribonucleic acid; UV, ultraviolet light; YPRE, photoreactivating enzyme from bakers' yeast; DPN, diphosphopyridine nucleotide.

DNA Native or denatured C_{25} DNA was extracted from *H. influenzae* resistant to cathomycin, 25 μg/ml.[2] In some experiments we tested native or denatured Sm_{250} DNA[3] (DNA extracted from *H. influenzae* resistant to streptomycin, 250 μg/ml) or E_{20} DNA[4] (DNA extracted from *H. influenzae* resistant to erythromycin, 20 μg/ml). Denatured DNA was obtained by heating at 100°C for 5 minutes and quenching in ice water. The DNA concentration during this study varied between 40 and 100 μg/ml.[5] After treatment with ultraviolet light, nitrous acid, or both, the denatured DNA was renatured by a method developed from that of Marmur, Schildkraut, and Doty (8) which involves heating for an hour at pH 7 and 66°C in the following manner: in general, a 10 to 1 ml sample of 2 to 5 μg/ml[5] of denatured DNA dissolved in 0.3 M sodium chloride–0.01 M sodium citrate was placed in a tube and incubated in a water bath at 65–67°C for 1 hour. Then the tube was transferred to 1 liter of water at 65–67°C and allowed to cool to 30°C which took about 2 hours. This procedure of heating for 1 hour and cooling slowly will for convenience be referred to as "annealing."

Ultraviolet Irradiation of Native or Denatured Transforming DNA For ultraviolet irradiation in general, DNA at a concentration between 40 and 100 μg/ml[5] in 0.15 M sodium chloride–0.01 M sodium citrate was exposed for various time periods at 45 cm to a 15 watt General Electric germicidal lamp with an output of approximately 25 ergs/mm²/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 or Buffer The method of Horn and Herriott (9) was followed in a general way. To 1 volume of a mixture (acetate buffer and 2 M $NaNO_2$ in 0.15 M saline) or buffer alone was added 1 volume of denatured or native DNA. The final concentration of $NaNO_2$ was 1 M, of buffer, 0.05 M, and the DNA was between 20 and 50 μg/ml;[5] the initial pH of this mixture was 4.8. It was observed by Boeye (10) and Horn and Herriott (9) that during incubation at 37°C the pH rose, probably by decomposition of nitrous acid. The reaction mixture was incubated at 37°C for 30 minutes, 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. It was then annealed as described above.

Dialysis The samples from nitrous acid or buffer treatment, previously neutralized with 0.02 M Na_2HPO_4 in 0.3 M saline and annealed, were dialyzed in the following manner: 1.5 ml of each sample was dialyzed against 2000 ml of 0.15 M sodium chloride–0.01 M sodium citrate at 5°C for 24 hours. The dialysis liquid was changed and the process continued until 144 hours of total time; the concentration of

[2] First isolated by Mary Jane Voll.

[3] This marker is Sm_α or Sm^{2000} of Hsu and Herriott (6) and was first isolated by Alexander and Leidy (7), but only 250 μg/ml of streptomycin was used in the present experiments for screening.

[4] This marker confers resistance to 20 μg/ml erythromycin, but only 15 μg/ml of antibiotic was used in the present experiments for screening.

[5] These differences of concentration of DNA are between experiments, not variations in the same experiment.

renatured C_{25} DNA during dialysis was 4 μg/ml. This dialysis is very important because some ingredient from the nitrous acid or buffer treatment, perhaps NO_2^-, interferes in some way with the photoreactivating enzyme.

Photoreactivation The photoreactivating enzyme from bakers' yeast (YPRE) (3) was used in this work. The photoreactivation mixtures consisted of 1.0 ml of DNA (dialyzed renatured DNA or native DNA) and 1.0 ml of 1:20 dilution in 0.15 M sodium chloride from stock YPRE;[6] the final concentration of DNA was 1 or 2 μg/ml.[5] The reaction mixtures were contained in screw-capped tubes and illuminated by a bank of three General Electric "black light" tubes (F20T12. BL, 20 watt emission between 300 and 400 mμ) at 37°C. The time of illumination in general was 60 minutes, which gave maximum photorecovery (see Fig. 8A); once the enzyme was added to the sample the mixture was either exposed to the reactivating light or stored in a lightproof container.

Transformation Procedure The assay procedure described by Goodgal and Herriott (5) was followed, but during the uptake of C_{25} DNA or Sm_{250} DNA a solution containing 0.1 M sodium chloride, 0.01 M phosphate buffer, and 0.02 per cent tween 80 at pH 7.0 was used instead of "Elev" broth, and then the overlaying method was followed; during the uptake of E_{20} DNA Difco brain-heart infusion was used instead of Elev broth, and then it was followed with the pour plate procedure. The titer in the reaction mixture was calculated and from this the per cent of residual transforming activity of the different samples relative to the control. This control in general consisted of a sample unirradiated with ultraviolet light and treated with the same buffer as the experimental sample but without nitrite, all this under the same conditions as the other samples.

New Transforming Markers In order to look for new transforming markers (9) in DNA after UV irradiation and nitrous acid or nitrous acid alone, the following assay was used: the reaction mixture consisted of 3.0 ml of Difco brain-heart medium (supplemented with hemin and DPN), 2×10^8 cells/ml, and 0.5 μg/ml of DNA. This mixture was shaken 150 minutes at 37°C. The cells were diluted with eugon-broth (Baltimore Biological Laboratory), and from the last dilution, plates were made of brain-heart agar plus hemin and DPN plus: (*a*) 25 μg/ml of cathomycin, (*b*) 4 μg/ml of kanamycin, (*c*) 5 or 250 μg/ml of streptomycin, and (*d*) 150 μg/ml of viomycin. After 24 to 48 hours of incubation at 37°C the colonies were counted. The results corrected for dilution before plating gave the number of mutants resistant to 25 μg/ml cathomycin, 4 μg/ml kanamycin, 5 or 250 μg/ml streptomycin, and 150 μg/ml viomycin.

[6] The stock YPRE is a purified fraction obtained from extracts of bakers' yeast using ammonium sulfate precipitation and column chromatography (11). Two ml of this preparation was dialyzed against 1000 ml 0.15 M NaCl–0.01 M sodium citrate at 5°C during 14 hours. This dialyzed sample was used immediately after dialysis.

EXPERIMENTAL RESULTS

Reactivation of Ultraviolet-Irradiated Denatured C_{25} DNA with Nitrous Acid

Denatured DNA was exposed to ultraviolet light for varying periods. The samples were divided into two series; to the control, buffer was added and to the other, buffer and nitrite. All the samples were neutralized, renatured, and tested for intrinsic C_{25} transforming activity. It can be seen in Fig. 1, that

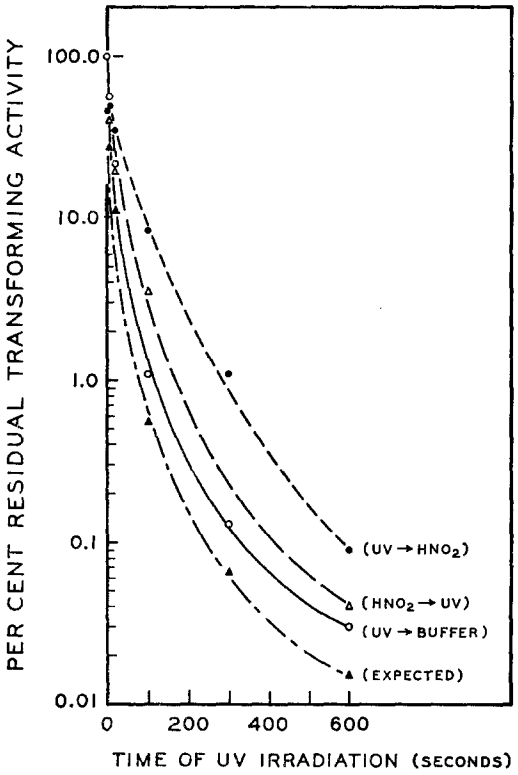

FIGURE 1. Nitrous acid reactivation of ultraviolet-irradiated denatured C_{25} DNA. UV irradiation, see the section on Methods. Concentration of denatured DNA, 100 μg/ml. HNO_2 or buffer treatment, 1 M nitrite in 0.05 M acetate buffer or the buffer alone; initial pH 4.8; concentration of denatured DNA 50 μg/ml; time, 30 minutes, temperature, 37°C. Expected curve, it was calculated as 50 per cent of the control (UV buffer) curve at each point (see text).

nitrous acid partially reversed the inactivation inflicted by ultraviolet irradiation. The values after nitrous acid treatment *were not corrected* for the inactivation produced by this reagent (about 50 per cent of the sample treated with buffer) although this correction could be justified since the reactivated marker is also sensitive to the exposure to nitrous acid (Fig. 5). Litman (12) and Horn (31) found a considerable drop in the uptake of nitrous acid–treated DNA by competent cells. These two corrections suggest that the reactivation of the ultraviolet-irradiated denatured DNA produced by nitrous acid is higher than shown in the curve in Fig. 1. The expected curve in this figure was calculated taking 50 per cent of the different

values of the samples treated with ultraviolet light and buffer; it represents the expected destructive effect of UV and nitrous acid together. These results confirm the earlier report (4) that nitrous acid partially restores UV-inactivated transforming DNA. Further evidence that this rise in C_{25} (and, as shown later, in Sm_{250}) marker is in fact a recovery of the intrinsic marker and not new markers induced by nitrous acid is suggested by the results mentioned in a footnote to Tables I and II that no C_{25} or Sm_{250} transforming

TABLE I

EFFECT OF UV IRRADIATION AND NITROUS ACID ON DENATURED Sm_{250} DNA

Samples Time of UV irradiation	Kanamycin (4 μg/ml) resistant mutants per ml mixture* Column I	Column II
sec. (no UV or HNO_2)	1.55×10^5	1.55×10^5
	UV → HNO_2	HNO_2 → UV
0	7.70×10^5	7.70×10^5
5	6.16×10^5	7.05×10^5
20	5.30×10^5	5.74×10^5
100	4.15×10^5	2.31×10^5
300	1.25×10^5	1.89×10^5
600	0.92×10^5	1.49×10^5

Column I, effect of UV irradiation on the formation of new markers with HNO_2.

Column II, sensitivity of the new markers produced by nitrous acid to UV irradiation.

* The number of cathomycin (25 μg/ml) resistant mutants was zero in all the samples.

Ultraviolet irradiation, described in the section on Methods. Concentration of denatured Sm_{250} DNA, 100 μg/ml in column I, 5 μg/ml in column II. Nitrous acid or buffer treatment, 1 M sodium nitrite in 0.05 M acetate buffer or the buffer alone, initial pH = 4.8. Concentration denatured Sm_{250} DNA, 50 μg/ml. Time, 30 minutes. Temperature, 37°C. Transformation mixture, 2×10^8/ml competent cells in brain-heart infusion, 0.5 μg/ml Sm_{250} DNA. Shake 150 minutes at 37°C.

markers were formed when nitrous acid acted on DNAs which initially did not carry these markers.

Studies of Some Factors Involved in the Nitrous Acid Reactivation of Denatured C_{25} DNA Inactivated with Ultraviolet Light

TEMPERATURE Samples of denatured DNA were UV-irradiated after which they were treated with buffer or nitrous acid at different temperatures for 30 minutes. Fig. 2 shows that the sample treated with buffer did not change its activity with temperature, but the recovery following nitrous acid

treatment increased between 24 and 36°C. Higher temperatures did not increase this reactivation further.

PH The results of experiments to determine the effect of the pH of the buffer-nitrite mixture on the reactivation are seen in Fig. 3. The samples treated with buffer alone showed no change in their activity, but reactivation produced by nitrous acid increased with increasing acidity below pH 5.4.

TABLE II

EFFECT OF UV IRRADIATION AND NITROUS ACID ON DENATURED E_{20} DNA

Samples Time of UV irradiation	Viomycin (150 μg/ml) resistant mutants per ml mixture* Column I	 Column II
sec. (no UV or HNO_2)	1.65×10^4	1.65×10^4
	UV → HNO_2	HNO_2 → UV
0	1.19×10^5	1.19×10^5
5	1.17×10^5	1.08×10^5
20	1.13×10^5	1.02×10^5
100	5.25×10^4	2.86×10^4
300	2.29×10^4	1.13×10^4
600	1.10×10^4	1.12×10^4

Column I, effect of UV on the formation of new markers with HNO_2.
Column II, sensitivity of the new markers produced by nitrous acid to UV irradiation.
* The number of streptomycin (250 μg/ml) resistant mutants was zero in all the samples.
Ultraviolet irradiation, described in the section on Methods. Concentration of denatured E_{20} DNA, 100 μg/ml in column I, 5 μg/ml in column II. Nitrous acid or buffer treatment, 1 M sodium nitrite in 0.05 M acetate buffer or the buffer alone, initial pH, 4.8. Concentration denatured E_{20} DNA, 50 μg/ml. Time, 30 minutes. Temperature, 37°C. Transformation mixture, 2×10^8/ml competent cells in brain-heart infusion, 0.5 μg/ml E_{20} DNA. Shake 150 minutes at 37°C.

SODIUM NITRITE CONCENTRATION Samples of UV-irradiated denatured DNA and the unirradiated controls were treated for a constant time with different concentrations of sodium nitrite in acetate buffer. The initial pH of the sample treated with 1 M sodium nitrite was 4.8. Fig. 4 contains the results. The activity of unirradiated samples fell with increasing nitrite until 0.25 molar was reached and beyond this there was no further change even though the concentration of nitrite was raised to 2.0 molar. In the samples with a prior exposure to ultraviolet light there was reactivation of genetic activity by the action of nitrous acid. The transforming titer increased almost linearly with nitrite concentration from 0.15 M to 1.0 molar and beyond this the increase was slower. Horn and Herriott (9) working with the same conditions found

that the initial pH of the reaction mixture was 4.2 to 4.7 when the concentration of sodium nitrite varied from 0.05 to 1.0 M. The results represented in Fig. 4 cannot be due to these changes of pH, because the ratio of the reactivation in 1 M to 0.05 M sodium nitrite is higher (>8.3) than the ratio of reactivation at pH 4.7 and pH 4.2 (about 1.5, Fig. 3). This means that the results obtained with different concentrations of sodium nitrite may be better correlated with the different concentrations of nitrous acid in the reaction mixture.

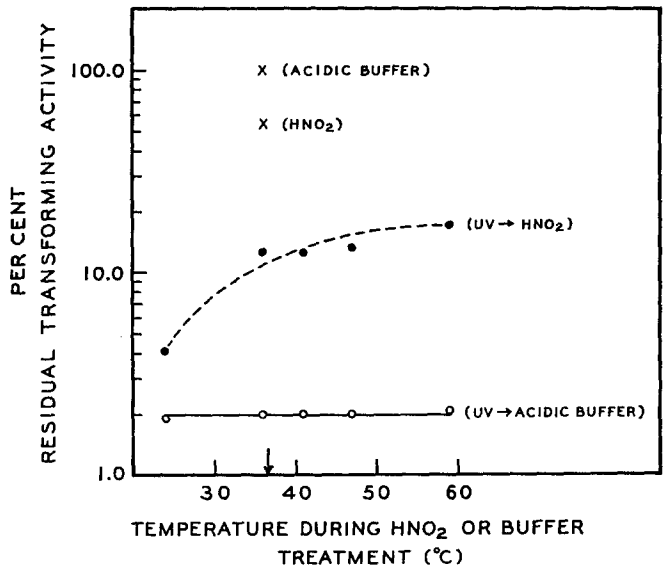

FIGURE 2. Effect of temperature on the nitrous acid reactivation of ultraviolet-irradiated denatured C_{25} DNA. UV irradiation, see the section on Methods. Concentration of denatured DNA, 40 μg/ml; time, 100 seconds. HNO_2 or buffer treatment, 1 M nitrite in 0.05 M acetate buffer or the buffer alone, initial pH 4.8; concentration of denatured DNA 20 μg/ml; time, 30 minutes, temperature as indicated in the figure. The upper signs (x) show the control values of samples without UV irradiation treated with buffer or HNO_2 at 36°C. The arrow over the temperature axis indicates the temperature at which most of the other experiments were made.

TIME The effect of time of exposure of the ultraviolet-irradiated denatured DNA to nitrous acid was determined. The results in Fig. 5 show that the samples treated with buffer in the absence of nitrite did not change their activity significantly during the different periods of incubation. The samples treated with nitrite were reactivated rapidly during the first 15 minutes and after that less rapidly up to 60 minutes at 37°C. Continued exposure to nitrous acid produced some inactivation. This inactivation produced by continuous exposure of the reactivated marker to nitrous acid is similar to that produced in unirradiated denatured DNA, seen in Fig. 5. A more detailed study of the

inactivation of denatured DNA produced by nitrous acid at different times of incubation was described earlier (9).

Effect of Nitrous Acid on Native C_{25} DNA Inactivated with Ultraviolet Light

When it was observed that nitrous acid partially reversed the damage produced by ultraviolet light in *denatured* C_{25} DNA, it was important to study this

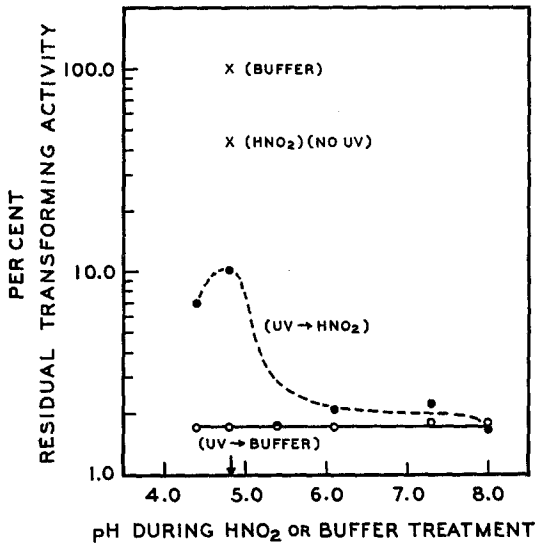

FIGURE 3. Effect of pH on nitrous acid reactivation of ultraviolet-irradiated denatured C_{25} DNA. UV irradiation, see the section on Methods. Concentration of denatured DNA, 40 μg/ml; time, 100 seconds. HNO_2 or buffer treatment, pH 4.4 to 6.1, 1 M nitrite in 0.05 M acetate buffer or the buffer alone; pH 7.3 and 8.0, 1 M nitrite in 0.01 M phosphate buffer or the buffer alone; initial pH is indicated in the figure, concentration of denatured DNA 20 μg/ml; time, 30 minutes, temperature, 37°C. After this the samples with pH between 4.4 and 6.1 were neutralized by a tenfold dilution with 0.02 M Na_2HPO_4 in 0.3 M NaCl. The samples at pH 7.3 or 8.0 were diluted tenfold with 0.01 M citrate in 0.3 M NaCl. The final pH oscillated between 7.2 to 7.6. The upper signs (x) show the control values of samples without UV irradiation treated with acetate buffer or HNO_2, pH 4.8. The arrow over the pH axis indicates the pH during most of the other experiments.

phenomenon with *native* DNA. Samples of native DNA inactivated with ultraviolet light and then treated with nitrous acid did not show any increase in comparison with the control in which no nitrite was used. On the contrary, the nitrous acid–treated samples showed less transforming activity than did the buffer controls. The unirradiated native DNA is more sensitive to the inactivation by nitrous acid than denatured DNA for in this case there was 20 per cent residual transforming activity and the unirradiated denatured DNA treated

under the same conditions showed nearly 50 per cent activity (Fig. 1). Perhaps this greater sensitivity accounts in part for the failure to reactivate UV-inactivated native DNA with nitrous acid.

Support for the notion that denaturation and renaturation have no effect on the nitrous acid recovery of UV damage was shown by an experiment in which the native DNA was first exposed to increasing doses of irradiation fol-

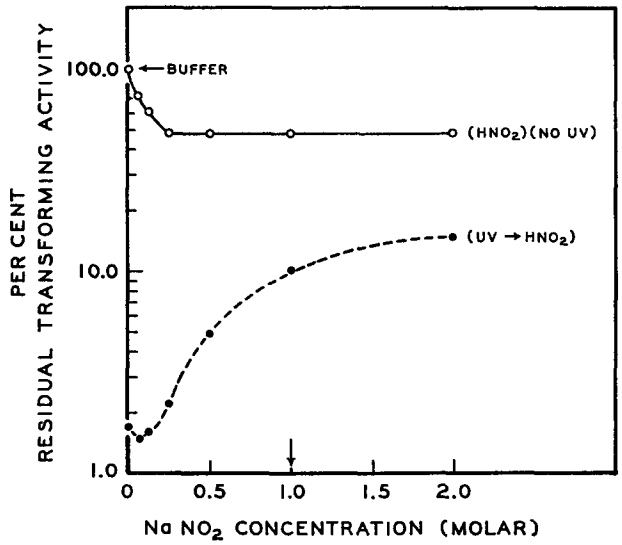

FIGURE 4. Nitrous acid reactivation of ultraviolet-irradiated denatured C_{25} DNA. Effect of the concentration of nitrite. UV irradiation, see the section on Methods. Concentration of denatured DNA, 40 μg/ml, exposure, 100 seconds. HNO_2 or buffer treatment, different concentration of nitrite in 0.05 M acetate buffer or the buffer alone; initial pH in the presence of 1 M nitrite was 4.8. Concentration of denatured DNA 20 μg/ml, time, 30 minutes, temperature, 37°C. The upper arrow shows the control value of a sample without irradiation treated with acetate buffer. The arrow over the concentration axis indicates the nitrite concentration at which most of the other experiments were made.

lowed by nitrous acid, then denatured, annealed, and assayed. The results of these experiments (not shown) indicate that nitrous acid failed to reactivate the UV inactivation of native DNA and denaturation followed by renaturation neither raised nor lowered the activity remaining after irradiation.

Nitrous acid pretreatment of denatured C_{25} DNA produced a slight protection against ultraviolet inactivation (Fig. 1) which was not seen in the case of native DNA. If we correct our curve for the inactivation produced by nitrous acid on an unirradiated sample of native DNA (in which the activity is reduced to about 15 per cent of the initial transforming activity) some protection against UV irradiation is suggested. Marmur *et al.* (13) made a similar

observation on the protection against ultraviolet irradiation afforded by pretreatment with nitrous acid of native pneumococcus Sm DNA.

Is the Nitrous Acid Reactivation Specific for Ultraviolet Damage of Denatured DNA?

From the experiments described above it is clear that nitrous acid reactivated ultraviolet damage in denatured DNA but there was no comparable reactiva-

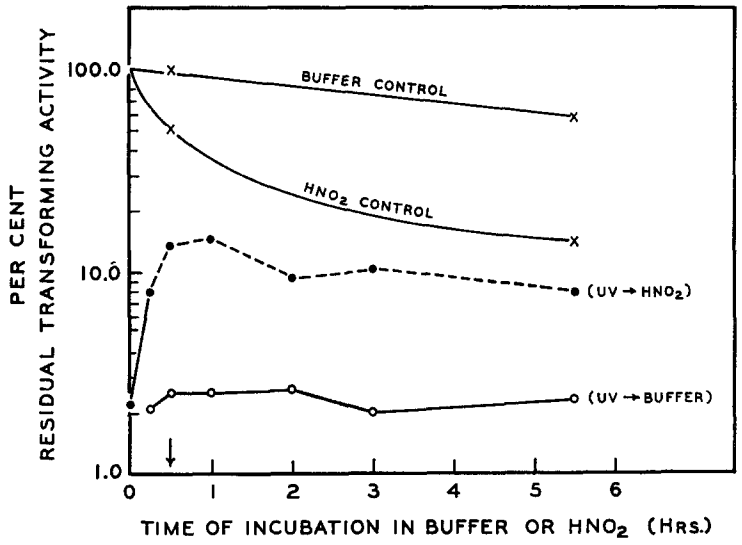

FIGURE 5. Nitrous acid reactivation of ultraviolet-irradiated denatured C_{25} DNA. Effect of time of incubation. UV irradiation, see the section on Methods. Concentration of denatured DNA 40 μg/ml, time, 100 seconds. HNO_2 or buffer treatment, 1 M nitrite in 0.05 M acetate buffer or buffer alone, initial pH 4.8, concentration of denatured DNA, 20 μg/ml, time is indicated in the figure, temperature, 37°C. The upper signs (x) show the control values of samples without irradiation treated with acetate buffer or HNO_2 during 0.5 or 5.5 hours. The arrow over the time axis indicates the time of treatment of samples in most of the other experiments.

tion in native DNA. These results can be interpreted in two ways: (*a*) the ultraviolet damage is different in native and denatured DNA, and (*b*) the UV damage is the same, but in the native form the damage cannot be reactivated by nitrous acid. In order to discriminate between these two possibilities, samples of native C_{25} DNA were irradiated for different periods of time, then the samples were denatured, treated with nitrous acid or buffer, and annealed. The results (Fig. 6A) show that after denaturation of ultraviolet-inactivated native DNA, the genetic transformations were increased by nitrous acid. In other experiments denatured DNA was inactivated with UV, then renatured, and the samples were treated with buffer or nitrous acid. In

this case no reactivation was observed; if the samples were reannealed after buffer or nitrous acid treatments the results were the same, showing that the renaturation process was not responsible for the observed difference. These experiments show that the nitrous acid–reactivable groups can be produced by ultraviolet irradiation of either native or denatured DNA but in order to be reactivated by nitrous acid, the reaction requires that the DNA be in the de-

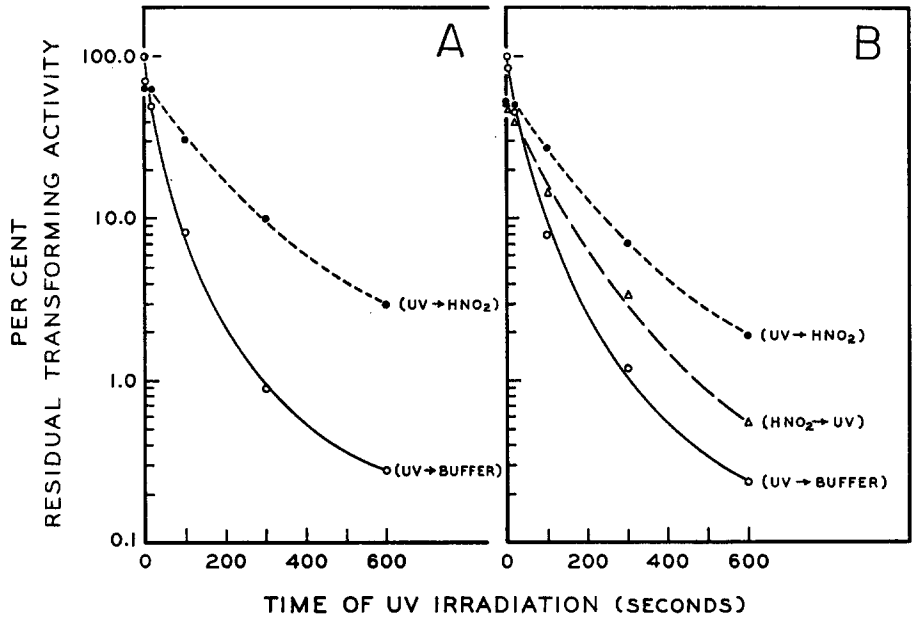

FIGURE 6A. Nitrous acid reactivation of ultraviolet-irradiated native C_{25} DNA denatured after irradiation. UV irradiation, see the section on Methods. Concentration of native DNA, 40 μg/ml. Denaturation, as described in the section on Methods. HNO_2 or buffer treatment, 1 M nitrite in 0.05 M acetate buffer or buffer alone, initial pH 4.8, concentration of denatured DNA 20 μg/ml; time, 30 minutes, temperature, 37°C.
FIGURE 6B. Nitrous acid reactivation of ultraviolet-irradiated denatured Sm_{250} DNA. UV irradiation, see the section on Methods. Concentration of denatured Sm_{250} DNA, 100 μg/ml. HNO_2 or buffer treatment, 1 M nitrite in 0.05 M acetate buffer or buffer alone. Initial pH, 4.8, concentration of denatured Sm_{250} DNA 50 μg/ml, time, 30 minutes, temperature, 37°C.

natured form, because the native or renatured DNA in some way protects the damage from the action of nitrous acid.

Nitrous Acid Reactivation of Ultraviolet Damage in Other Markers

Thus far the studies have been limited to the C_{25} marker. To determine the extent to which this phenomenon might be marker-specific other markers were studied. In Fig. 6B it can be seen that similar to C_{25} DNA, denatured

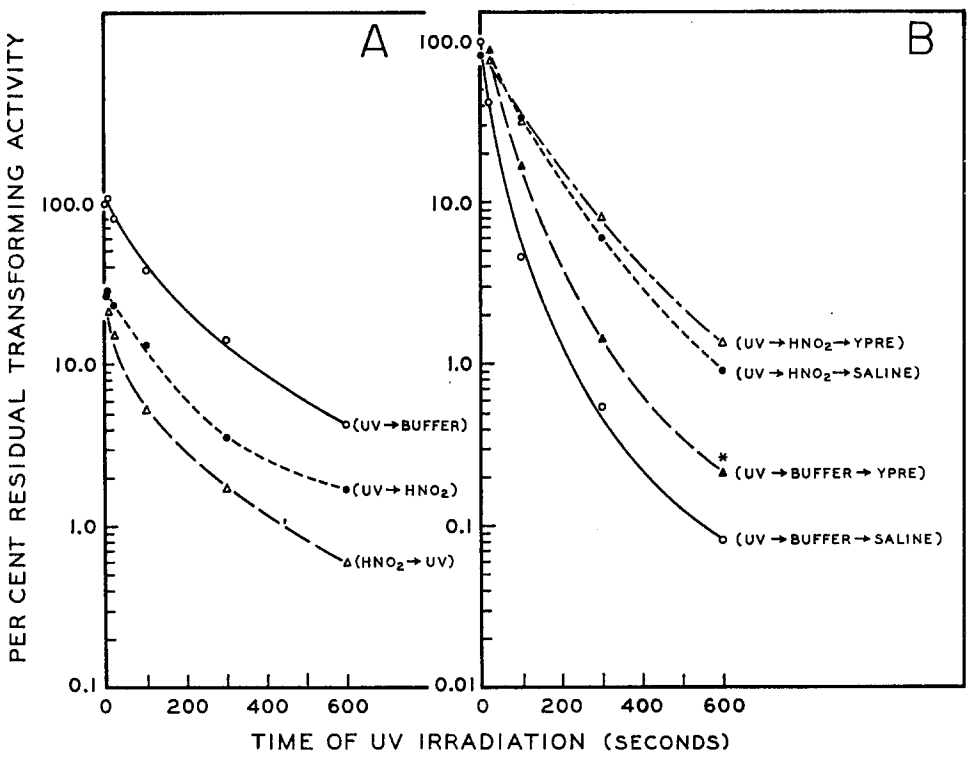

FIGURE 7A. Nitrous acid on ultraviolet-irradiated denatured E_{20} DNA. UV irradiation, see the section on Methods. Concentration of denatured E_{20} DNA, 100 μg/ml. HNO_2 or buffer treatment, 1 M nitrite in 0.05 M acetate buffer or buffer alone, initial pH 4.8, concentration denatured E_{20} DNA, 50 μg/ml, time, 30 minutes, temperature, 37°C.

FIGURE 7B. Nitrous acid and photoreactivation of ultraviolet-irradiated denatured C_{25} DNA. UV irradiation, see the section on Methods. Concentration of denatured C_{25} DNA, 80 μg/ml. HNO_2 or buffer treatment, 1 M nitrite in 0.05 M acetate buffer or buffer alone, initial pH 4.8, concentration of denatured DNA, 40 μg/ml, time, 30 minutes, temperature, 37°C. Dialysis, the samples from HNO_2 or buffer treatment were neutralized with 0.02 M Na_2HPO_4 in 0.3 M saline and they were annealed (this is the usual procedure described in Methods); then 1.5 ml of each sample was dialyzed against 2000 ml of 0.15 M NaCl–0.01 M sodium citrate at 5°C, at 24 hours the dialysis liquid was changed and the dialysis continued until 144 hours of total time; concentration of renatured DNA, 4 μg/ml. Photoreactivation (YPRE treatment), as described in the section on Methods; concentration of dialyzed renatured DNA 2 μg/ml. Saline treatment, to these samples was added 0.15 M NaCl instead of YPRE and they were illuminated in the same conditions indicated in the photoreactivation procedure; concentration of dialyzed renatured DNA, 2 μg/ml.

* A duplicate of this sample was made, but to it after neutralization was added $NaNO_2$ (0.1 M final concentration); this sample after renaturation, dialysis, and photoreactivation gave the same value of residual activity as the sample without $NaNO_2$ added.

Sm_{250} DNA inactivated with ultraviolet light was reactivated by nitrous acid and again the pretreatment with nitrous acid of "single stranded" (denatured) DNA protected somewhat against ultraviolet irradiation.

In the case of E_{20} DNA a completely different picture was obtained (Fig. 7A). The denatured marker inactivated with ultraviolet light was not reactivated with nitrous acid. The curves resemble the inactivation produced by UV plus the corresponding inactivation produced by nitrous acid of an unirradiated sample. When the denatured E_{20} DNA was pretreated with nitrous acid, it was sensitized to the ultraviolet irradiation, and these samples showed the highest inactivation. A similar behavior was obtained with *native* E_{20} DNA (Table III).

TABLE III

NITROUS ACID EFFECT ON ULTRAVIOLET-IRRADIATED NATIVE E_{20} DNA

Samples treated with	Per cent of resistant transforming activity
Buffer	100
HNO_2	8.2
UV → buffer	34.3
UV → HNO_2	4.1
HNO_2 → UV	3.7

Ultraviolet irradiation, described in the section on Methods. Concentration of E_{20} DNA, 100 μg/ml. Time, 100 seconds. Nitrous acid or buffer treatment, 1 M sodium nitrite in 0.05 M acetate buffer or buffer alone, initial pH, 4.8. Concentration of E_{20} DNA, 50 μg/ml. Time, 30 minutes. Temperature, 37°C.

Relation between Nitrous Acid Reactivation and Photoreactivation

When nitrous acid reactivation of ultraviolet damage in DNA was established, it became of interest to determine the possible relationship between this reactivation and the reactivation produced by the photoreactivating enzyme (1–3). With this in mind the next experiments were performed. Samples of denatured C_{25} DNA were inactivated with ultraviolet light and then they were treated with nitrous acid. After renaturation and dialysis, some samples were treated with 0.15 M sodium chloride and others with the photoreactivating enzyme from bakers' yeast (YPRE). The record of the results is presented in Fig. 7B. It can be seen that the reactivation produced by YPRE alone is less than the reactivation produced by nitrous acid alone; samples which were first reactivated with nitrous acid and then with YPRE showed the highest reactivation. In the samples UV-irradiated 300 or 600 seconds the reactivation was about the sum of the nitrous acid reactivation plus the photoreactivation. In other experiments similar to this the photoreactivation alone

was higher than in the present experiment, but it was not higher than the reactivation produced by nitrous acid alone.

Two samples of denatured C_{25} DNA were irradiated with UV during 300 seconds and treated with buffer or nitrous acid. After renaturation and dialysis they were treated with 0.15 M saline or YPRE respectively and incubated in

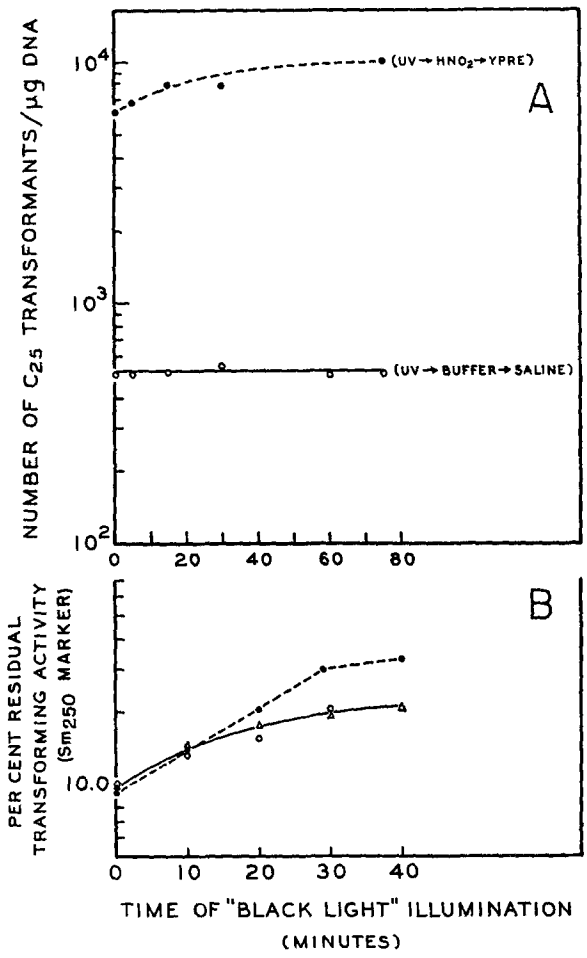

FIGURE 8A. Photoreactivation after nitrous acid reactivation conditions of this experiment. See the legend of Fig. 7B.

FIGURE 8B. Competitive inhibition of photoreactivation by nitrous acid–reactivated DNA. UV irradiation, see the section on Methods. Concentration of native Sm_{250} DNA, 80 μg/ml. Time, 100 seconds. UV C_{25} DNA and HNO_2 reactivated UV C_{25} DNA, for details in the preparation of these samples see legend of Fig. 7B and section on Methods. Photoreactivation (YPRE treatment), see the section on Methods. Concentration of either DNA, 1 μg/ml.

●, UV Sm_{250} DNA + YPRE, △, UV Sm_{250} DNA + UV C_{25} DNA + YPRE, ○, UV Sm_{250} DNA + HNO_2 reactivated UV C_{25} DNA + YPRE.

the presence of black light for different times. The results in Fig. 8A show that after nitrous acid treatment the sample was reactivated about tenfold in comparison with the control. In the presence of YPRE this sample was further reactivated during incubation in the presence of black light. At about 60 minutes a plateau level was reached.

Two samples of denatured C_{25} DNA were inactivated by 100 seconds of ultraviolet irradiation and treated with buffer or nitrous acid, after which the samples were renatured and dialyzed. These samples were analyzed for their "competitive inhibition" (25) of YPRE during the photoreactivation of native Sm_{250} DNA inactivated with 100 seconds of ultraviolet irradiation. The results in Fig. 8B indicate that the competitive inhibition of the UV-irradiated C_{25} DNA did not change following nitrous acid treatment for it gave the *same competitive inhibition* during the photoreactivation of the UV-inactivated Sm_{250} DNA as the control which received no nitrous acid treatment.

All these findings suggest that the ultraviolet damage reactivated by nitrous acid is different from that reactivated by the photoreactivating enzyme from bakers' yeast and that these two reactivations are roughly additive.

Photoreactivation of irradiated denatured DNA has been reported by Marmur and Grossman (14) and confirmed by us (15). This supports Rupert's earlier observations (16) that irradiated denatured DNA competes favorably for the photoenzyme from yeast. In the present paper the enzymic photoreactivation took place after the irradiated denatured DNA was renatured. Setlow (17) reported no photoreactivation of irradiated native DNA which was denatured and renatured before exposure to the photoenzyme. In repeating Setlow's experiments using *Hemophilus* DNA we observed a two- to threefold increase as a result of photoenzymic treatment.

DISCUSSION

The reactivation of ultraviolet-irradiated DNA with nitrous acid requires the single stranded (denatured) form, but the UV irradiation can be applied to either denatured or native DNA. This strictly chemical reactivation is affected by certain factors, such as: temperature, pH, nitrous acid concentration, and time of incubation. It is not affected by illumination with light of wave length of 3400 to 3500 A, which is required for photoreactivation (1–3). This new reactivation might be explained by the (*a*) formation of new markers, or (*b*) reactivation of UV damage. In relation to the first possibility it is known (9) that nitrous acid forms antibiotic resistance markers in denatured DNA but in the experiments described no high level antibiotic resistance markers were observed so this mechanism will not explain the present case. The alternative explanation, namely reversal of inactivation, fits more nearly the evidence obtained thus far.

The studies of ultraviolet irradiation of purines and pyrimidines, bases,

nucleosides, nucleotides, and desoxyribonucleic acid (for reviews see 13, 17, and 18) suggest that the changes produced in DNA include: (*a*) alteration of pyrimidine bases (the 1, 4, addition of water to the thymine moiety (19), photochanges of the cytosine moiety), (*b*) "inter-" or "intra-" crosslinks (thymine dimers) (20–22), formamide and heat-stable interstrand linkages (13, 14, 23, 24), and (*c*) certain backbone breakage.

Which, if any, of these possibilities is involved in the changes found to be reversible by nitrous acid and which, if any, is reversed by the yeast photo-

TABLE IV

EFFECT OF UV IRRADIATION AND NITROUS ACID ON DENATURED C_{25} DNA

Samples Time of UV irradiation	Streptomycin (5 μg/ml) resistant mutants per ml mixture Column I	 Column II
sec. (no UV or HNO_2)	2.30×10^4	2.30×10^4
	UV → HNO_2	HNO_2 → UV
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

Column I, effect of UV irradiation on the formation of new markers with HNO_2.

Column II, sensitivity of the new markers produced by nitrous acid to UV irradiation.

Ultraviolet irradiation, described in the section on Methods. Concentration of denatued C_{25} DNA, 100 μg/ml in column I and 5 μg/ml in column II. Nitrous acid or buffer treatment, 1 M sodium nitrite in 0.05 M acetate buffer or the buffer alone, pH, 4.8. Concentration of denatured C_{25} DNA, 50 μg/ml. Time, 30 minutes. Temperature, 37°C. Transformation mixture, 2×10^8/ml competent cells in brain-heart infusion, 0.5 μg/ml C_{25} DNA. Shake 150 minutes at 37°C.

reactivating enzyme? It is indicated in the present paper that these two reversing procedures do not overlap so it may be tentatively assumed that the changes in these two cases are different.

The effect of pH and nitrite concentration on the deamination of bases in T2 bacteriophage DNA or pneumococcal transforming DNA (26, 27, 12) compared to these effects on the reversal of UV damage in transforming DNA suggests that the latter is not brought about by deamination. The deamination is much more strongly pH-dependent than is the reversal. On the other hand the inactivation of markers by nitrous acid (presumably due to deamination) was less affected by increasing the nitrite concentration above 0.25 M whereas

the reactivation rose linearly up to 1.0 molar nitrite. This, then, suggests only that the nitrous acid reversal of UV damage to DNA is not expected to be a deamination.

The failure of nitrous acid to reactivate directly native DNA inactivated with UV may be due to cross-linking produced by nitrous acid (28, 29) or to masking of essential groups in the double helix structure, or both.

The difference between the nitrous acid reactivation of UV damage in Sm_{250} DNA and C_{25} DNA is not great but the failure to reactivate E_{20} DNA is difficult to explain. It is not due to gross differences among the samples of DNAs, because the three behaved similarly during the formation of nitrous acid–induced genetic markers (Tables I, II, and IV). The E_{20} DNA marker is more resistant to UV irradiation (compare Fig. 7A with Figs. 1 and 6B) than C_{25} DNA or the linked markers (30) Sm_{250} DNA and $C_{2.5}$ DNA.

The protection against ultraviolet irradiation of denatured C_{25} DNA or Sm_{250} DNA and the sensitization of denatured E_{20} DNA by pretreatment with nitrous acid need more experimentation. When Marmur *et al.* (13) observed that nitrous acid treatment of native pneumococcal DNA reduced the subsequent effects of UV, it was suggested that this was due perhaps to a similarity of the lesions produced by the two treatments. If this were true, the order of treatment would probably not be important. Our results on denatured DNA show that the order of treatment is quite important for it was this that led to the observation that nitrous acid partially reversed UV damage.

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