Photodynamic Action of Proflavine on Coliphage T3

III. Damages to the Deoxyribonucleic Acid Associated with Rx1 and Rx2

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Ultracentrifugal studies with deoxyribonucleic acid (DNA) extracted from phage exposed to light in the presence of either 0.25 or 8.5 μg of proflavine per ml reveal that the lethal damage of Rx1 renders DNA alkali-labile, with lethality resulting from damage that occurs singly on either strand or simultaneously on both strands. Apparently nonlethal damages temporally associated with Rx1 consist of (i) heat and alkali-labile cross-links (which produce undenatured DNA that migrates at 45S) and (ii) heat-labile bonds. The formation of 45S material is a linear function of light dose, and the production of this material ceases when Rx2 appears at the higher dye concentration. No tendency to plateau is seen at 0.25 μg of dye per ml. The nature of the lethal damage of Rx2 could not be determined. Damages that were temporally associated with Rx2 at 8.5 μg of dye per ml were heat-labile, alkali-stable cross-links (undenatured DNA, 38 to 41S; alkali-denatured-reenutralized DNA, 85 to 89S) and some double-stranded breaks. No such changes were seen at 0.25 μg of dye per ml.

Despite the fact that both proteins and nucleic acids are sensitive to photodynamic action, most evidence strongly suggests that phage lethality results mainly from alteration of the nucleic acid moiety (5–8, 19).

Reports do exist on the effect of photodynamic action on phage deoxyribonucleic acid (DNA) in vitro (14) and on infectious tobacco mosaic virus ribonucleic acid (RNA; references 9, 15). There is, however, only one report of the effects of photodynamic action on nucleic acids extracted from phage that have been exposed to inactivating conditions. This study (5) demonstrates that the kinetics of inactivation of T7 are biphasic in the presence of 1 μg of acridine orange per ml. Presumably, these reactions correspond to Rx1 and Rx2 for T3 and proflavine (20). Freielder and Uretz found that, as long as Rx1 alone is proceeding, the only observable damage introduced into T7 DNA is the formation of alkali-labile bonds. At higher doses of light (less than 1% survival), other kinds of damage are seen (e.g., cross-linking, single- and double-strand breaks).

The present communication deals with the damage introduced into the T3 chromosome when the phage is exposed to light in the presence of either 0.25 or 8.5 μg of the dye proflavine per ml.

MATERIALS AND METHODS

Saline-ethylenediaminetetraacetic acid (EDTA) was composed of 0.1 M NaCl and 0.001 M EDTA, pH 7.8 (3).

Neutral formaldehyde consisted of 100 μl of 10^{-2} M NaOH per 100 ml of 36.8% HCHO. All other materials were as described in the preceding papers (20, 21).

Boundary centrifugation of DNA from illuminated phage. Four-milliliter samples of phage (2×10^{11} to 4×10^{11}/ml) and dye were placed in an open petri dish. A 10-μl amount was removed for plating. The remainder was illuminated for the desired period of time, and another 10 μl was removed for plating. The rest was pipetted into dialysis tubing (Union Carbide Corp., Chicago, Ill.) and dialyzed at 0 C for 24 hr against four changes (60 volumes each) of saline-EDTA.

DNA was released by heating T3 in saline-EDTA for 5 min at 65 to 70 C (3). One-milliliter samples were then treated in the following ways. One sample, not treated further, was considered to be native DNA. A second sample was heated at 95 C for 5 min, 0.08 ml of neutral formaldehyde was added, heating was continued for an additional 30 sec, and then the sample was immersed in an ice bath. This constituted heat-denatured DNA. The third sample was cooled to room temperature after the DNA had been released. Then 0.04 ml of 8.0 M NaOH was added, raising the pH to 12.3; the DNA was kept at this pH for 5 min after which time 0.1 ml of 1.0 M KH_{2}PO_{4} and 0.07 ml of 4.2 M HCl were added to bring the pH down to about 8. Routinely, wide-range pH paper (B. D. H. Wide Test Paper) was used to monitor the reneutralization step. This was alkali-denatured reneutralized DNA (referred to hereafter as "alkali-denatured DNA").

All centrifugation was done in the Spinco model E analytical ultracentrifuge, equipped with ultraviolet optics. The samples were centrifuged in a single-sector aluminum cell at 33,450 rev/min.
The sedimentation value was corrected to that expected in a solution with the density and viscosity of water at 20°C in the usual way.

**Thermal denaturation profiles of T3 DNA.** Thermal denaturation profiles for T3 DNA were obtained in saline-EDTA. A Beckman DU spectrophotometer, equipped with a heating chamber and thermal spacers, was used. Temperature was controlled with a Colora ultrathermostat (model N-27694; Papst, Germany)

Phage at \(2 \times 10^9\text{ml}^{-1}\) (ca. 20 \(\mu\text{g}\text{ml}^{-1}\) of DNA), in 1.5% phosphate buffer, was dialyzed into saline-EDTA. Three-milliliter samples were placed in matched, plastic-stoppered cuvettes. The temperature was raised to 70°C for 5 min to release the DNA and then the temperature was increased at set intervals. After the heating chamber had come to a given temperature, an additional 3 min was allowed before the optical density was determined. Optical densities were corrected for the expansion of water.

**RESULTS**

**Untreated DNA: shape of boundary.** Native T3 DNA showed a single homogeneous boundary upon centrifugation (Fig. 1). On the other hand, denatured DNA species exhibited biphasic boundaries. There was a leading front which was sharply defined, followed by a more diffuse tail region. The amount of trailing material was 50% with both heat-denatured and alkali-denatured DNA (3) and was independent of both ionic strength and DNA concentration.

**Untreated DNA: sedimentation coefficients.** T3 DNA has the following sedimentation coefficients \((S_{20, w})\): native DNA, 34 ± 1; heat-denatured DNA, front, 32 ± 1; tail, 26 ± 1; alkali-denatured DNA, front, 74 ± 1; tail, 68 ± 1.

With the formula derived by Studier (17), native T3 DNA has a molecular weight of 28 \(\times\) 10^8 daltons.

**Controls.** No alteration in any of the above parameters was observed with DNA extracted from phage illuminated in the absence of dye or exposed to dye in the dark. Extracting the phage DNA by the standard phenol technique had no effect on the above parameters or the results described below. Recent studies indicate that reducing agents produce single-stranded breaks in DNA. It has been found that cysteine, which was used as a radical trap in the kinetic experiments described in the preceding paper (21), is no exception under the conditions used for these studies. The results indicate that breaks begin to appear immediately after adding cysteine. Within 1 hr, at 18°C, all strands have received a break but no phage lethality is detectable. Due to these findings, definitive studies on the effects of cysteine on the quantity of damage produced by photodynamic action have been deferred until conditions can be developed which obviate this effect of cysteine on DNA.

DNA extracted from phage exposed to light and proflavine: native DNA. After illumination in the presence of 0.25 or 8.5 \(\mu\text{g}\) of proflavine per ml, undenatured DNA no longer sedimented as a single component, but rather as two components. A faster, minor boundary sedimented at 45S, whereas a major more slowly moving boundary behaved like normal native DNA, except at low survival levels at 8.5 \(\mu\text{g}\) of dye per ml (Fig. 2). With 0.25 \(\mu\text{g}\) of proflavine per ml (Fig. 2a and b), the amount of faster-moving DNA increased linearly with light dose at a rate described by the equation \(P_{45} = 0.27T\) (Fig. 2b; Table 1). \(P_{45}\) is the percentage of DNA at 45S, \(T\) is the illumination time in minutes, and 0.27 is a constant). At 8.5 \(\mu\text{g}\) per ml (Fig. 2c and d), the situation

![Fig. 1. Boundary centrifugation of DNA extracted from untreated T3. (a) Native DNA; (b) heat-denatured DNA; (c) alkali-denatured DNA. In all figures showing boundary centrifugation, the direction of centrifugation is to the left and the numbers in parentheses correspond to the minutes of centrifugation.](http://jvi.asm.org/)

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Optical density upon shown DNA corrected was equipped after the plastic-stoppered Three-milliliter DNA denatured in ultrathermostat (model N-27694; Papst, Germany). Phage at \(2 \times 10^9\text{ml}^{-1}\) (ca. 20 \(\mu\text{g}\text{ml}^{-1}\) of DNA), in 1.5% phosphate buffer, was dialyzed into saline-EDTA. Three-milliliter samples were placed in matched, plastic-stoppered cuvettes. The temperature was raised to 70°C for 5 min to release the DNA and then the temperature was increased at set intervals. After the heating chamber had come to a given temperature, an additional 3 min was allowed before the optical density was determined. Optical densities were corrected for the expansion of water.

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was more complex. There was an initial linear increase in 45S material with doses as high as 13 min of illumination (2 phage lethal hits), after which no further increase was detectable (Fig. 6c). The equation defining the linear region of the dose-response curve is \( P_{45} = 1.15 T \).

The slower-moving major boundary always migrated around 35S when phage were illuminated with 0.25 \( \mu \text{g} \) of the dye per ml (Table 1). With 8.5 \( \mu \text{g} \) of proflavine per ml, this was so only for doses lower than 25 min (5.5 phage lethal hits). At this and higher doses, the 35S boundary was replaced completely by a homogeneous boundary which migrates somewhat more rapidly (Table 2). No boundaries migrating at intermediate values were detectable.

No double-strand breaks were seen at 0.25 \( \mu \text{g} \) of proflavine per ml. Double-strand breaks did appear at high doses of light and 8.5 \( \mu \text{g} \) of dye per ml (Fig. 2d, Table 2).

**DNA extracted from phage exposed to light and proflavine: heat-denatured DNA.** With 0.25 \( \mu \text{g} \) of dye per ml and doses of light of 5 min or less, heat-denatured DNA gave boundaries and sedimentation coefficients (Fig. 3a) identical to those obtained for heat-denatured untreated DNA (Fig. 1b). However, at between 5 (0.33 phage lethal hit) and 10 min (0.67 phage lethal hit) of illumination, a rapid, short-lived reaction occurred. From 10 min of illumination onward, 20% of the heat-denatured DNA migrated as an apparently homogeneous boundary at 26S. The remainder sedimented as a broad polydisperse boundary with an average \( S_{20, w} \) of 23 (Fig. 3b, c).

A similar reaction occurred at 8.5 \( \mu \text{g} \) of dye per ml (Fig. 3d,e). With exposure times as low as 0.1 min, the reaction was well under way. The front migrated at 30S, unlike the situation at 0.25 \( \mu \text{g} \) of dye per ml.

### Table 1. Effect on native DNA of illumination of T3 in the presence of 0.25 \( \mu \text{g} \) of proflavine per ml

<table>
<thead>
<tr>
<th>Time of illumination (min)</th>
<th>Lethal hits</th>
<th>( S_{20, w} ) of slower boundary</th>
<th>Per cent slower boundary</th>
<th>Per cent 45S, w, material</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>34</td>
<td>100</td>
<td>0</td>
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<tr>
<td>7</td>
<td>0.47</td>
<td>35</td>
<td>89</td>
<td>11</td>
</tr>
<tr>
<td>20</td>
<td>1.33</td>
<td>34</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td>25</td>
<td>1.67</td>
<td>35</td>
<td>87</td>
<td>13</td>
</tr>
<tr>
<td>30</td>
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<td>35</td>
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</tr>
<tr>
<td>50</td>
<td>3.33</td>
<td>36</td>
<td>83</td>
<td>17</td>
</tr>
<tr>
<td>70</td>
<td>4.66</td>
<td>33</td>
<td>76</td>
<td>24</td>
</tr>
<tr>
<td>90</td>
<td>6.00*</td>
<td>35</td>
<td>79</td>
<td>21</td>
</tr>
<tr>
<td>110</td>
<td>7.35*</td>
<td>35</td>
<td>70</td>
<td>30</td>
</tr>
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</table>

* These survival levels were determined by extrapolation of the best line through the points obtained with the lower exposure times. Rx2 definitely does not occur at exposure times equal to or less than 70 min. Data presented in the preceding paper indicate that, under these conditions, Rx2 probably does occur, at a low rate, at exposure times greater than 70 min. Therefore, the survival levels shown for 90 and 110 min of illumination are probably for Rx1 alone. Per cent double-stranded breaks for all times of illumination was 0.
proflavine per ml, whereas the average sedimentation coefficient of the tail region was 23S, as at the lower dye concentration. The rate of formation of these heat-labile bonds was linear with dose between 0.1 min and roughly 13 min of illumination and the slope of the line was 4% per min (Fig. 6d, Table 3). At present, it is impossible
to state what the kinetics are before 0.1 min. With exposure times equal to or greater than 13 min,
there was no discrete boundary which sedimented at 30S, but the leading edge of boundary did
diminate at this rate.

When double-strand breaks began to show in native DNA, the average S20,w of the heat-denatured
dNA was reduced slightly and the boundary became a bit broader. Notwithstanding, the leading
ege of the boundary still migrated at 30S.

**DNA extracted from phage exposed to light and proflavine: alkali-denatured DNA.** Upon alkali
denaturation, DNA extracted from viruses exposed to inactivating conditions showed a per cent
increase in trailing material that can be well correlated with the per cent dead phage produced by
Rx1 (Fig. 4). Comparison of the per cent dead phage versus per cent increase in trailing material
(damaged strands) produced by alkali-treatment (Fig. 5a, 6a) and the per cent increase in trailing
material (damaged strands) produced upon alkali denaturation as a function of illumination
(Fig. 5b, 6b) gives excellent agreement with the theoretical curves expected if lethality in Rx1
results from damage which occurs on one DNA strand or the other or simultaneously on both.

At 0.25 µg/ml, the front moved at 74S20,w.

**TABLE 2. Effect on native DNA of illumination of T3 in the presence of 8.5 µg of proflavine
per ml**

<table>
<thead>
<tr>
<th>Time of illumination (min)</th>
<th>Lethal hits*</th>
<th>S20,w of slower boundary</th>
<th>Per cent slower boundary</th>
<th>Per cent 45S20,w material</th>
<th>Per cent double-stranded breaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>34</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.125</td>
<td>35</td>
<td>97</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0.375</td>
<td>36</td>
<td>96</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0.625</td>
<td>35</td>
<td>93.5</td>
<td>6.5</td>
<td>0</td>
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<tr>
<td>7</td>
<td>0.875</td>
<td>35</td>
<td>92.5</td>
<td>7.5</td>
<td>0</td>
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<tr>
<td>8.5</td>
<td>1.05</td>
<td>34</td>
<td>90.3</td>
<td>9.7</td>
<td>0</td>
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<tr>
<td>10</td>
<td>1.25</td>
<td>34</td>
<td>88.5</td>
<td>11.5</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>2.00 (1.63)</td>
<td>36</td>
<td>85</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>2.30 (2.00)</td>
<td>35</td>
<td>83</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>4.00 (2.50)</td>
<td>35</td>
<td>81.5</td>
<td>18.5</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>5.50 (3.13)</td>
<td>39</td>
<td>78</td>
<td>17</td>
<td>5</td>
</tr>
<tr>
<td>30</td>
<td>7.00 (3.75)</td>
<td>38</td>
<td>81</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>40</td>
<td>10.50 (5.00)</td>
<td>41</td>
<td>75</td>
<td>16</td>
<td>9</td>
</tr>
</tbody>
</table>

* Numbers in parentheses refer to the lethal hits due to Rx1. The unenclosed numbers are the
  total lethal hits.

(Fig. 3). This was also the case for 8.5 µg/ml and light doses below 25 min of illumination
(5.5 phage lethal hits). At this and higher doses, the front migrated more rapidly. Nonetheless,
the amounts of trailing material observed agreed well with those predicted by the either strand model
for the particular survival levels expected if Rx1 alone were proceeding (Table 5).

**DISCUSSION**

DNA extracted from T3 exposed to light and proflavine: native DNA. When DNA is extracted
by mild heat shock from phage which have been
The chemical nature of this cross-link has not been determined. The bond is labile to both heat and alkali, since no corresponding boundaries are seen upon denaturation.

The apparent cross-linking produced at high doses of light and 8.5 μg of dye per ml is different. It causes no alteration in the amount of 45S material, but it affects all other molecules to the same extent, as evidenced by the homogeneity of the boundary. Its $S_{20,w}$ is 38 to 41, implying that this DNA is slightly less compact than the 45S material. The reaction is fairly rapid; all molecules not traveling at 45S are converted to 38 to 41S mate-

![Image](http://jvi.asm.org/)
Table 3). Major bonds induced by alkali-denatured DNA are broken at 8.5 μg of dye per ml, and the reaction is apparently complete within 5 min.

With 8.5 μg of dye per ml, double-stranded breaks are seen at survival levels equal to or less than 5.5 phage lethal hits. Even after 10.5 phage hits, less than 10% of the molecules are broken (Fig. 2d; Table 2). Double-stranded breaks are probably lethal (4) but, under the conditions employed here, these appear fairly late and are never seen in significant amounts. Clearly, double-stranded breaks are not a primary lethal event.

DNA extracted from T3 exposed to light and proflavine: heat-denatured DNA. The heat-labile bonds induced by light and proflavine cannot be the major lethal damage of Rxl either (Fig. 3, Table 3). At 0.25 μg/ml, the reaction is restricted to a span of time no longer than 5 min. With 8.5 μg/ml, most of the heat-labilizing reaction is confined to the first 6 sec of illumination (Fig. 6d), in which time few phages are killed.

At both 0.25 and 8.5 μg of dye per ml, the polydisperse heat-denatured trailing material migrates at 23S. From these data, it may be concluded that in either case a strand that is damaged receives the same number of heat-sensitive bonds and these altered bonds are located the same distance apart.

Although the average sedimentation rate of the trailing material is the same at both dye concentrations, the fronts migrate at different rates. With 0.25 μg of dye per ml, the front travels at 26S (half-sized strands), but at the higher concentration of dye the front moves at 30S (indistinguishable from intact strands).

At each dye concentration, when the amount of trailing material in the front has been reduced to 20% of the total DNA concentration, the reaction undergoes a pronounced change. There is no further production of heat-labile bonds with 0.25 μg of dye per ml, and the rate of production of heat-labile bonds decreases sharply in the experiments with 8.5 μg/ml. In both instances, it would seem there is an initial increase in radical density sufficient to drive the reaction to a certain point, followed by a decrease in radical density. At the lower concentration of dye, this reduced radical density is insufficient to maintain the reaction, whereas there is a sufficiently high radical density at 8.5 μg of dye per ml, to enable the production of heat-labile bonds to proceed, albeit at a slower rate.

The small changes in S20, w observed between intact strands and damaged strands imply that the resulting strand pieces are large, that is, relatively few nucleotides are so affected as to give heat-
FIG. 6. Kinetics of production of various types of damage at 8.5 μg of proflavine per ml. (a) Per cent dead phage produced by Rx1 as a function of per cent increase in trailing material upon alkali denaturation. The theoretical curves are the same as those presented in Fig. 5. (b) Per cent increase in trailing material upon alkali denaturation as a function of minutes of illumination in the presence of 8.5 μg of dye per ml; the line is that expected from the either strand model. (c) Per cent native DNA which migrates at 45S as a function of minutes of illumination in the presence of 8.5 μg of proflavine per ml. (d) Per cent increase in trailing material upon heat denaturation as a function of minutes of illumination at 8.5 μg of dye per ml.

labile bonds. Models show that all of the observed sizes of damaged strands can be accounted for with two heat-labile bonds per originally broken strand and three altered bonds per originally intact strand.

Nothing definite can be said now about the chemical nature of the heat-labilizing damage. It is known that alkylation agents render the DNA backbone heat-labile via the production of triesters (16) which are formed by adding an alkyl function to the phosphodiester group, but it is difficult to see how photodynamic action could produce analogous damage.

DNA extracted from T3 exposed to light and proflavine: alkali-denatured DNA. Although the production of 45S material and heat-sensitive bonds is associated temporally with Rx1, it is unlikely that either represents the major lethal damage of this reaction. However, the generation of alkali-labile bonds can be correlated with the production of dead phage by Rx1 via a realistic model.

Three theoretical mechanisms that seem plausible are represented by the either strand, particular strand, and both strands models. The pertinent equations are based on the assumptions that both
strands of the DNA duplex are equally susceptible to the production of alkali-labile bonds and the two strands behave independently. As a result, the frequency with which the individual strands become alkali-labile is identical to the frequency with which the total number of strands become alkali-labile.

The either strand model predicts that lethality results from damage which occurs singly on either strand or simultaneously on both. The particular strand model predicts that lethality results from damage to a particular strand, and the both strands model predicts that the lethality occurs only when damage is present on both DNA strands. The equations are given in the legend to Fig. 5.

The data presented here favor the either strand model for both dye concentrations tested (Fig. 5a,b; 6a,b; Tables 4, 5). Furthermore, these data reveal that reactions involving different radical mechanisms can generate the same damage, and the number of alkali-labile bonds required to kill a phage remains unaltered. At 0.25 μg of dye per ml, Rx1 is composed of at least two parallel first-order reactions, one cysteine-sensitive and the other cysteine-insensitive; with 8.5 μg of proflavine per ml, the former operates for the first minute only, at most (21).

Recent studies with ribonucleosides show that an early reaction is the rupture of the N-glycosidic bond of guanosine, yielding ribose and the free base (10). Within DNA, deoxyguanylate appears to be the nucleotide residue most affected but some deoxthyminylate is also destroyed (6, 7, 14). If the lethal reaction of Rx1 leads to partial depurination or depyrimidation, or both, the appearance of alkali-labile bonds on the DNA of phage exposed to light and dye is explained. Both apurinic and apyrimidinic acids are alkali-labile (2, 12, 13).

Other experiments with DNA show that most of the photoproducts of deoxyguanylate remain attached to the sugar-phosphate backbone (11). This damage could be nonlethal or it could labilize the N-glycosidic bond to alkali-treatment in which case the sugar-phosphate backbone would become alkali-labile too.

The question remains as to whether this damage constitutes a replicational or transcriptional block. Studies indicate that only one strand of the T7 DNA molecule is transcribed (18). A similar situation should exist in T3. Were the damage that leads to alkali lability a transcriptional block, the data should have followed the particular strand model, unless damage to the nontranscribed strand can block reading which seems unlikely. Consequently, it seems best to conclude, tentatively, that generation of alkali-labile bonds leads a replicational block.

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LITERATURE CITED

