Characteristics of the Photoreactivation of Pseudorabies Virus

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ABSTRACT

PFEEFFERKORN, E. R. (Harvard Medical School, Boston, Mass.), BOYCE W. BURGE, AND HELEN M. COADY. Characteristics of the photoreactivation of pseudorabies virus. J. Bacteriol. 92:856-861. 1966.—Pseudorabies virus, killed with ultraviolet light and then allowed to adsorb to chick embryo tissue culture cells, can be photoreactivated by light of longer wavelengths. The photoreactivable sector is approximately 0.5. Herpes simplex virus is equally photoreactivable. Photoreactivability is negligible during the first 20 min after adsorption and then rises to a maximum by 100 min. After this maximum is reached, photoreactivability decreases with increasing rate as the temperature of incubation is raised. Inhibition of protein synthesis immediately after irradiation prevents the establishment of the photoreactivable state.

In many microbial systems, including bacteria, bacteriophage, and fungi, lethal damage by ultraviolet (UV) light can be reversed through subsequent exposure to light of longer wavelengths [reviewed by Jagger (6)]. Detailed study of this photoreactivation has led to important observations on the photochemistry and enzymology of deoxyribonucleic acid (DNA). Photoreactivation studies with cells of higher animals have not been as profitable, as viability is restored only in complex systems such as the whole mouse (12).

We describe here a simple system for the demonstration of photoreactivation of a DNA-containing animal virus in tissue culture cells. We initially observed that UV-treated pseudorabies virus allowed to adsorb to chick embryo tissue culture cells could be revived by exposing the cell-virus complex to light of longer wavelengths (10). Since the cell-virus complexes were part of a monolayer, the photoreactivated viruses went on to form plaques. This report describes some aspects of the establishment and loss of the photoreactivable state.

MATERIALS AND METHODS

Tissue culture and viruses. Primary chick embryo fibroblasts in monolayer cultures were used in all experiments. Plaque titrations of pseudorabies virus and herpes simplex virus were done by the methods described for Sindbis virus (9), except that the cultures were stained with neutral red 4 days after infection. All cultures were prepared in soft-glass bottles.

Pseudorabies virus grown in chick embryos was obtained from Luis Melendez. Stocks of about 3 × 10^8 plaque-forming units (PFU)/ml were prepared in chick tissue cultures and stored at -60 C.

Herpes simplex virus, obtained from Joan Daniels as an extract of infected chick chorioallantoic membrane, was used without subsequent passage.

Ultraviolet irradiation. A 15-w Westinghouse "germicidal" lamp was the ultraviolet source. Virus stocks were diluted with cold Hanks balanced salt solution lacking phenol red until their absorbancy at 260 mp was negligible; then, they were irradiated in a watch glass with the lamp at 27 cm. After the addition of calf or rabbit serum to a final concentration of 5%, the inactivated stocks were stored at -60 C.

Our experiments employed virus inactivated to a surviving titer of 10^{-3} to 10^{-4} of the original. Photoreactivation of such UV-treated stocks produced a 20- to 30-fold increase in titer (10). This increase corresponded to a photoreactivable sector of 0.5. The photoreactivable sector is the fraction of the total cross section for ultraviolet irradiation that is subject to photoreactivation [defined by Dulbecco (4)].

Photoreactivation. Two sources of photoreactivating light were used. Most experiments employed a General Electric "photo reproduction" fluorescent lamp (F 48PG17-1 PR) that has a broad emission maximum from 300 to 420 mp, the spectral range known to be most active in microbial photoreactivation (7). In a room with a temperature of 36 C, this lamp gave off sufficient heat to raise the temperature of treated cultures to 38 C. Maximal photoreactivation (photoreactivable sector of 0.45 to 0.55) was obtained by exposure to this lamp at 8 cm for 20 min or at 20 cm for 90 min. In experiments in small incubators, a small Westinghouse "blacklight" lamp that did not significantly raise the temperature was employed.
It should be noted that these special lamps are not essential for the demonstration of photoreactivation. Our preliminary experiments (10) were done with an ordinary white fluorescent lamp.

Photoreactivation in the first 2 hr of infection was generally done in liquid medium; when the period of photoreactivation extended into the 3rd hr of infection, cultures were covered with agar before irradiation. This eliminated the possibility that newly released virus would initiate more plaques. Photoreactivation was equally efficient in liquid medium and under agar. Cultures containing liquid medium were irradiated from below. Cultures covered with agar were irradiated from above with the photoreactivating light passing first through the glass. Soft glass has an acceptable transmittance of 30 to 85% in the spectral range of 320 to 420 μm.

Radioactivity. Incorporation of C\textsuperscript{14}-leucine (Schwarz Bioresearch, Orangeburg, N.Y.) into tissue cultures was determined by dissolving the monolayer in 0.3 N NH\textsubscript{4}OH and by precipitating the acid-insoluble material with cold 0.3 N trichloroacetic acid. The precipitate was washed well with cold 0.3 N trichloroacetic acid, dissolved in 0.3 N NH\textsubscript{4}OH, dried on aluminum planchets, and counted in a gas-flow counter.

RESULTS

Establishment of the photoreactivable state. Preliminary experiments showed that UV-treated virus could not be photoreactivated immediately after adsorption. The time required for the appearance of maximal photoreactivability was therefore determined. Suitable dilutions of UV-treated pseudorabies virus were allowed to adsorb to replicate monolayer cultures at 4 C. After 1 hr, the cultures were rapidly warmed by several rinses with medium and then were incubated at 37 C with a liquid medium. At intervals, cultures were exposed to a saturating dose of photoreactivating light and then covered with agar. Other cultures received a subsaturating dose of photoreactivating light to determine whether the dose requirement remained constant during the period when the photoreactivability was increasing. Figure 1 shows that the photoreactivability was negligible during the first 20 min after adsorption and then rapidly rose to a maximum at 100 min.

Photoreactivation by the subsaturating dose of light remained a reasonably constant fraction of that produced by the saturating dose. Thus, the dose required for maximal photoreactivation remained constant throughout the period when photoreactivability was increasing. Thus, the increase must represent the transition of the cell-virus complex into a state of potential photoreactivability.

Since no photoreactivation occurred when monolayers received photoreactivating light between 0 and 20 min after adsorption, the phenomenon of "photoprotection," sometimes observed in bacterial systems (18), may be ruled out.

Decline of the photoreactivable state. The stability of the photoreactivable state was determined at several temperatures: 18, 28, 36, and 42 C. Pseudorabies virus is able to form plaques over the temperature range from 33 to 41 C (Pfefferkorn and Rutstein, unpublished data). Virus treated with UV was allowed to adsorb to monolayer cultures in dim light at room temperature for 1 hr. The cultures were then covered with agar and incubated at 36 C for 2 hr. As stated above, this is sufficient time for the establishment of maximal photoreactivability. Sets of cultures were subsequently moved to incubators at the other temperatures. At intervals, these cultures were rapidly warmed or cooled to 36 C and either kept dark or treated with a saturating dose of photoreactivating light. The cultures were then incubated for 4 days at 36 C.

Whereas the titers of cultures kept dark were generally not affected by the change in temperature during incubation, the titers of cultures kept at 42 C for more than 12 hr declined. Thus, at 42 C only the first 12 hr could be studied. Figures 2 and 3 show that the stability of photoreactivable centers is markedly dependent on incubation temperature: at 42 C the half-life of these centers is less than 2 hr, whereas at 28 C it is 15 hr.

The rates at the four temperatures yield a linear
Arrhenius plot (Fig. 3); from the slope of this plot, an activation energy of about 30 kcal/mole can be computed. This value is somewhat greater than that found for most enzymatic reactions. The nature of the reaction that leads to the loss of photoreactivability is unknown.

**Effect of temperature on the process of photoreactivation.** The temperature dependence of the process of photoreactivation was examined over the temperature range from 31 to 41 C. Adsorption of UV-treated virus was carried out at room temperature, and the cultures were covered with agar and incubated at 36 C for 1.75 hr to allow the establishment of maximal photoreactivability. Sets of cultures were then transferred to incubators at other temperatures for exposure to photoreactivating light. After 0.5 hr, to allow for temperature equilibration, they were treated with photoreactivating light from a General Electric "blacklight" lamp. The cultures were then returned to 36 C for 4 days of incubation. In this experiment, it was essential to use a dose of photoreactivating light that was subsaturating: 2-hr exposure at a distance of 18 cm. For comparison, a saturating dose from the usual source of photoreactivating light was supplied to replicate cultures. Table 1 shows that the subsaturating dose of photoreactivating light yielded a progressively smaller photoreactivable sector as the temperature was lowered. Thus, photoreactivation is a temperature-dependent reaction.

**Protein synthesis and the establishment of photoreactivability.** If photoreactivability could be established in the absence of protein synthesis, any active participation of the viral genome in the process would be eliminated. To test this possibility, we used the antibiotic cycloheximide (The Upjohn Co., Kalamazoo, Mich.), which causes a rapid but reversible inhibition of protein synthesis in animal cells (5).

First, it was necessary to show that the presence of cycloheximide did not inhibit the process of photoreactivation once maximal photoreactivability was established. Cultures with UV-treated virus adsorbed were incubated at 36 C for 1.5 hr with liquid medium to establish a maximal state

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**Table 1. Effect of temperature on photoreactivation**

<table>
<thead>
<tr>
<th>Temp during photoreactivation</th>
<th>Source of photoreactivating light</th>
<th>Apparent photoreactivable sector</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 36</td>
<td>General Electric photo reproduction lamp (saturating dose)</td>
<td>0.50</td>
</tr>
<tr>
<td>41</td>
<td>General Electric &quot;backlight&quot; lamp (subsaturating dose)</td>
<td>0.42</td>
</tr>
<tr>
<td>36</td>
<td>General Electric &quot;backlight&quot; lamp (subsaturating dose)</td>
<td>0.34</td>
</tr>
<tr>
<td>31</td>
<td></td>
<td>0.22</td>
</tr>
</tbody>
</table>

* Cultures were first incubated at 36 C for 1.75 hr to establish a state of maximal photoreactivability.
of photoreactivability. Then some of the cultures received cycloheximide (8 \( \mu \)g/ml), and others were covered with agar. Some bottles from each set were exposed to a saturating dose of light; others were kept dark. The cycloheximide-treated cultures were immediately washed several times with warm medium and then covered with agar. The presence of cycloheximide at this stage of infection did not affect either the dark or the photoreactivated titer. Thus, it could be validly used to study the establishment of photoreactivability.

Replicate cultures were treated with cycloheximide (8 \( \mu \)g/ml) for 0.5 hr at 36 C. Then suitable dilutions of UV-treated virus were allowed to adsorb at 4 C for 1 hr. The cultures were washed three times with warm medium and incubated at 36 C in medium with or without cycloheximide. After a further 1.5 hr, the cultures incubated without cycloheximide were covered with agar. At 1.5 to 3 hr after adsorption, some cultures from each group received a saturating dose of photoreactivating light at 20 cm, while others were kept dark. After the illumination, the cultures containing cycloheximide were washed three times with warm medium and then covered with agar.

Other cultures carried through the same procedure were incubated with medium containing \( ^{14}C \)-leucine (10^6 counts per min per \( \mu \)mole) during the 1.5 hr immediately after adsorption. The presence of cycloheximide during this period reduced the incorporation of the labeled amino acid to 4.5% of the control.

The results of two independent experiments (Table 2) show that the presence of cycloheximide did not affect the titer in cultures kept dark. However, photoreactivation was markedly inhibited (to 6.6% of the control value). Thus, protein synthesis in the first 1.5 hr of infection appears necessary to establish the state of photoreactivability.

**Photoreactivation of other viruses containing DNA.** We examined another virus of the herpes group to see whether photoreactivation was only characteristic of pseudorabies virus. Figure 5 compares the UV sensitivity and the photoreactivability of herpes simplex virus with those of pseudorabies virus. These two viruses behaved similarly; each virus showed a photoreactivable sector of about 0.5. It is likely that photoreactivability is a property of the herpesviruses.

Not all DNA viruses, however, exhibit photoreactivation in chick embryo tissue cultures. We

![Graph](image-url)

**Fig. 4. Arrhenius plot of the rates of loss of photoreactivability in Fig. 2 and 3. The rates were calculated in days^-1.**

<p>| Table 2. Effect of inhibition of protein synthesis on establishment of photoreactivability |
|---------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Treatment with cycloheximide (78 ( \mu )g/ml)</th>
<th>Treatment with photoreactivating light^a</th>
<th>PFU/ml ( \times 10^4 )</th>
<th>Photoreactivated FFU/ml ( \times 10^4 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>For 0.5 hr before adsorption of virus only</td>
<td>None (dark)</td>
<td>4.1, 6.5(^c)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>From 1.5 to 3 hr after adsorption</td>
<td>78, 79</td>
<td>73</td>
</tr>
<tr>
<td>For 0.5 hr before adsorption of virus and during the first 3 hr after adsorption</td>
<td>None (dark)</td>
<td>4.2, 4.9</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>From 1.5 3 hr after adsorption</td>
<td>10.2, 8.4</td>
<td></td>
</tr>
</tbody>
</table>

^a Photoreproduction lamp at 20 cm.

^b Photoreactivated titer minus the dark titer.

^c Values from independent experiments.

^d This value corresponds to a photoreactivable sector of 0.53.
have described negative results with vaccinia virus (10) which are explained by Joklik's (8) finding that poxviruses exposed to UV light are not "uncoated" in cells to which they adsorb.

**Discussion**

The photoreactivation of pseudorabies virus resembles in several ways the photoreactivation of bacteriophage T2, described in detail by Dulbecco (4). The rate of photoreactivation of bacteriophage T2 showed a Q10 of 2 over the temperature range from 16 to 37°C (4). Our data do not permit a precise calculation of Q10, but the process of photoreactivation of pseudorabies virus was temperature-dependent. Ultraviolet-treated bacteriophage remains photoreactivable if the infected cells are kept cool, but, under normal growth conditions, photoreactivability is rapidly lost (4). Similarly, we found that photoreactivable centers (cell-virus complexes) were rapidly lost at high temperatures but were much more stable at 18°C.

Bowen (1) observed that the maximal rate of photoreactivation is not achieved until 8 min after the attachment of bacteriophage T2. The time required to establish maximal photoreactivability of pseudorabies virus is about 100 min. This longer time may represent, at least in part, the slower uncoating and penetration of this virus.

The mechanism of photoreactivation in microbial systems has been described by Wulff and Rupert (19) and Setlow (14). The most common lethal damage to DNA is the formation of intrastrand thymine dimers (16). During photoreactivation, these dimers disappear from the DNA (19). It is probable that dimers are split *in situ* by the photoreactivating enzyme (14), in contrast to the excision of dimers which occurs during dark repair of UV damage (2, 15).

A few instances of photoreactivation in higher animals have been reported [reviewed by Jagger (6)]. These, however, dealt with complex phenomena, e.g., the restoration of limb-bud regeneration (11) or the revival of the UV-irradiated newborn mouse (12). Any detailed experiments on such systems would be difficult.

In contrast, the search for photoreactivation in the simple system afforded by tissue cultures has yielded negative results. Chu (3) found no photoreactivation of UV-induced chromosomal aberrations of Chinese hamster cells. Cleaver (Biophys. Soc. Abstr., p. 111, 1966) found no photoreactivation of the UV-induced inhibition of DNA synthesis in tissue culture cells. We know of no positive reports of the photoreactivation of the viability of tissue culture cells. Cleaver (*personal communication*) has found no evidence of such photoreactivation in two tissue culture cell lines. Trosko et al. (17) labeled Chinese hamster cells in culture with H3-thymidine to detect the formation of thymine dimers by the UV-irradiation. The dimers were formed with the same efficiency seen in microbial systems, but there was no loss of dimers after irradiation with visible light. Thus, neither the phenomenon of photoreactivation nor its proposed biochemical mechanism could be demonstrated in an experimental system that might have been expected to detect them.

In contrast, our experiments have clearly demonstrated photoreactivation in animal cells. Several explanations could account for this difference. First, this phenomenon may occur in chick cells but not in mammalian cells. Second, the formation of thymine dimers may not be the critical lethal event in the UV irradiation of animal cells, which may possess some more UV-sensitive target. The UV-treated virus, however, should contain dimers which might be split by a repair enzyme in the undamaged cell. Finally, the viral genome, itself, might contain genetic information for a photoreactivating enzyme absent in the cell.

The observation that inhibition of protein synthesis after infection also inhibits photoreactivation is consistent with active participation of the viral genome in the phenomenon. However,
two alternative explanations should be considered. First, the photoactivating enzymes may be cellular in origin but be induced by the presence of thymine dimers in the UV-treated viral DNA. Second, it is possible that the cellular photoactivating enzymes are located in the nucleus and that the viral DNA must penetrate there to be repaired. This penetration may be blocked in the absence of protein synthesis.

Since the enzyme(s) involved in photoreactivation can be detected with a cell-free system, and since methods exist for measuring the disappearance of thymine dimers during photoreactivation (15), it should be possible to determine the role of the cellular and viral genomes in this example of photoreactivation in animal cells.

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LITERATURE CITED