Retardation of Regeneration and Division of *Blepharisma* by Ultraviolet Radiation and Its Photoreversal

ARTHUR C. GIESE and MOLLY LUSIGNAN

From the Department of Biological Sciences, Stanford University

**Abstract** Regeneration of *Blepharisma undulans* variety *japonicus* from which the hypostome has been removed is retarded by dosages of 3000 to 4600 ergs/mm$^2$ at wavelength 2654A most strongly when the fragment is exposed soon after cutting. Dosages greater than 4600 ergs/mm$^2$ prevent regeneration. Regeneration is also retarded strongly when the *Blepharisma* are cut soon after irradiation. Starvation retards regeneration and potentiates the effect of ultraviolet radiations. Division after regeneration of *Blepharisma* is also retarded by ultraviolet radiations about equally, regardless of when the *Blepharisma* are cut indicating a more lasting effect of the radiations upon the cells. *Blepharisma* cut after irradiation usually recover from the effects of the radiations sooner than uncut individuals given the same dosage. Retardation of division by ultraviolet radiation is subject to photoreversal by visible light, especially in a nitrogen atmosphere, provided the ultraviolet dose is not excessive. Visible light alone if prolonged, retards regeneration or may even kill the cut fragments of *Blepharisma*.

When the hypostome of a *Blepharisma* is cut off it is regenerated within about 5 or 6 hours (Moore, 1924). If the remaining fragment is exposed to ultraviolet (UV) radiations regeneration of the hypostome takes considerably more than 5 hours, the delay depending upon the wavelength and the dosage of the radiations (Hirshfield and Giese, 1953). Recent studies have shown that the regeneration-retarding effect of x-rays upon regeneration of *Blepharisma* is also dependent to a considerable extent upon the time at which the fragments are irradiated after cutting. Similar experiments using UV radiations as well as correlated effects upon division of the animals and photoreversal of UV injury by visible light are reported below.
MATERIALS AND METHODS

*Blepharisma undulans*, variety *japonicus* (Suzuki, 1954) was used in this study because of its large size and consequent ease of handling. The cultures were grown in lettuce infusion on a single strain of bacteria (*Pseudomonas ovalis*) and handled in a manner similar to that reported in previous papers (Giese, 1938; Giese and Lusignan, 1960). The cytostome, including all the mouth organelles, was removed from each of a number of individuals in excess of the number required for an experiment, by a single slice across the peristomial field, cutting the animals approximately in half. Twelve posterior fragments of about the same size were used as controls and sixteen were isolated in a quartz cell and irradiated. Twelve of the latter were later transferred to fresh lettuce medium previously inoculated with bacteria in the twelve depressions of a Kline agglutination slide, as were also the controls on another slide. Each slide was moist-chambered in a sterile Petri dish which was placed in a larger moist chamber kept in a constant temperature room at 25°C. When determinations of division rates were to be made after regeneration was complete, the *Blepharisma* were added to small test tubes of lettuce medium in the manner described for *Paramecium* (Giese, 1945). Observations for regeneration were made at hourly intervals until regeneration was complete in 50 per cent of the individuals (end point), after which they were made daily on cultures in test tubes until the animals had divided at least three times.

A d.c. quartz mercury arc running at atmospheric pressure and about 350 volts served as the source, the radiations of which were passed through a quartz monochromator with which the wavelength 2654 Å was isolated. This wavelength was chosen because a previous study had shown that it was quite effective in delaying regeneration (Hirshfield and Giese, 1953). The radiations were initially measured by a thermopile as in previous studies (Giese et al., 1952). Subsequently, however, a photovoltaic photocell calibrated against a thermopile was used to measure the intensity. The amplified photocell current trips a mechanical counter which records the dosage. The photocell system has the advantage over the thermopile of speed of operation as well as the capacity to integrate variations in intensity which were found to occur with current fluctuations. The intensity of the ultraviolet radiation at wavelength 2654 Å was found to range from 4.6 to 15.1 ergs/mm²/sec. for the various experiments reported in this paper. The intensity of the light declined gradually with use of the arc, principally because of devitrification of the quartz arc. When flashed UV was used it was obtained by interposing a disc of black paper with a sector cut out of it in each 180° and it was run at a speed of 450 r.p.m. The speed was measured with a strobotac.

For photoreactivation studies cultures were exposed 1 foot from the light of two 90 watt fluorescent daylight lamps for either 30 minutes or 1 hour. All cultures were handled and observed in yellow light which is inactive in photoreactivation of protozoans (Giese, 1953), care being taken especially after irradiation.

Usually at least three series of experiments were performed to test each point. When

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experiments were not fully consistent more were carried out. Since the action of radiations on *Blepharisma* varies from day to day (perhaps because of variations in the nutritional state of the animals), for valid comparisons it was felt necessary to perform a complete series of experiments on a given day to test all the particular points under consideration.

**EXPERIMENTAL**

1. **Fragments Irradiated at Various Times after Cutting** When the fragments are irradiated at $\frac{1}{2}$, 1, 2, 3, and 4 hours after cutting, the effectiveness of the UV in delaying regeneration declines with lapse of time since cutting, as shown in Fig. 1. This indicates that whatever reactions may have been initiated by cutting are well under way and become progressively less susceptible to UV injury. By 4 hours after cutting the regeneration of the fragments is only slightly affected by UV radiations.

Although regeneration is only slightly affected by UV radiation when the *Blepharisma* are exposed 4 hours after cutting, the animals are nonetheless, injured by the radiation. This is demonstrated by the delayed division subsequent to irradiation. As seen in Fig. 2, for three series of experiments, the...
effect of UV on division is much the same regardless of the time after cutting in which the fragments were exposed to the radiations, the delay in division of those exposed \( \frac{1}{2}, 1, 2, 3, \) and \( 4 \) hours after cutting being essentially alike, although the *Blepharisma* appear to be somewhat more sensitive to UV \( \frac{1}{2} \) hour and 3 hours after cutting. Division and regeneration, therefore, are differentially sensitive to UV, as they are to x-rays (Giese and Lusignan, 1960).

It is interesting to note (Fig. 2) that the irradiated cut fragments of animals recover from UV damage and ultimately divide at a rate faster than do uncut animals irradiated with the same dosage as shown by the shortening of the gap between divisions. This is true even though before they can divide, they must first reconstitute the protoplasm lost by removing the hypostome. This phenomenon was observed consistently in all sets of experiments performed, resembling in this respect the results of similar studies with x-rays (Giese and Lusignan, 1960). If the time required to reconstitute the hypostome is subtracted from the time to the first division of cut animals, the contrast between the rate of recovery of irradiated cut and irradiated uncut animals is even greater.

2. Fragments Cut at Various Times after Irradiation Regeneration of *Blepharisma* cut at various times after irradiation, namely 15 minutes, 2, 4 and
8 hours was retarded most in animals cut soonest after irradiation. This suggests that some recovery occurs within 2 hours. Since those cut 2 and 8 hours after irradiation showed about the same degree of regeneration delay, it seems likely that the initial process of recovery from irradiation reached its climax within 2 hours, after which only a second phase of very slow recovery occurs, as shown in Fig. 3.

Division of irradiated fragments is retarded to much the same degree regardless of when they are cut—15 minutes, 2, 4, or 8 hours after irradiation; the small differences in sensitivity which occur are not observed consistently. However, when the division of irradiated and cut Blepharisma is compared with the division of those which have been irradiated but not cut, it is found that cutting irradiated Blepharisma stimulates quicker recovery from radiation injury, as judged by later divisions of the cells. Cutting an unirradiated Blepharisma does not make it divide faster than an uncut control—in fact,
division is delayed until the protoplasm removed has been reconstituted. Cutting by itself, therefore, does not provide a stimulus to division, only cutting before or after irradiation. The effect shows up more strikingly when the data are plotted as in Fig. 4 rather than as in Fig. 2.

3. Effect of Starvation upon Regeneration If stores of food reserves are used in the regeneration process one might expect that a starved Blepharisma lacking such reserves would regenerate a lost part more slowly than would a well-fed one. This was indeed found to be the case, as shown in Fig. 5. If the effect of UV is specific upon some particular nutrients or upon some synthetic system required for regeneration, one might expect additional retardation following irradiation. This is seen in Fig. 5 and resembles the effect of x-rays on starved Blepharisma (Giese and Lusignan, 1960). Because Blepharisma does not grow on a synthetic medium it is not possible to correlate this change in resistance to UV with any specific nutrient at the present time.

4. Effects of Flashing Ultraviolet Light upon Division Flashing UV retards division of Didinium nasutum more than does continuous light of the same dosage (Giese et al., 1956). Because of its theoretical implications, the experiment was tried with Blepharisma; the results are shown in Fig. 4. A more powerful effect of flashing light is evident, especially for cut animals.
5. Photoreversal of UV-Treated Blepharisma

Several attempts were made to reverse the retarding effect of UV radiations on regeneration of *Blepharisma* by illumination with visible light. *Blepharisma* treated with UV, then cut and placed in Kline slides under fluorescent light (with a heat shield of 5 cm. of water in a plastic dish and a Corning filter No. 3389 which cuts off between 4160 and 4360 A), for 30 minutes or an hour, were delayed as much (or more) as those not illuminated. Illumination in nitrogen was also ineffective. If *Blepharisma* fragments were illuminated for as long as it took for completion of regeneration in the unilluminated UV-treated animals, they were invariably injured or killed.

Therefore, the effect of visible light, only, on regeneration was tested. Cut *Blepharisma* illuminated in a number of experiments with an intensity of visible light between 100 and 250 foot-candles, filtered free of heat and long UV as in the experiment described above, cytolyzed during the illumination at some time between 2 to 12 hours of exposure.

Next, tests were made with still lower intensities of light, 74 foot-candles, using the heat filter and Corning No. 3389 filter as above. Illumination continued until regeneration was complete, 12 hours after cutting, as shown in

![Figure 5. Effect of starvation on regeneration of *Blepharisma*. Histogram to show the effect of 3 days of starvation on regeneration and irradiation sensitivity of *Blepharisma*. Dosage 4600 ergs/mm². The solid lines indicate an experiment in which the effects were most marked. Usually the regeneration occurred much sooner (except in the control) as indicated by the dotted lines.](image-url)
FIGURE 6. Effect of visible light (intensity 74 foot-candles) upon regeneration and division of Blepharisma. The light from a daylight fluorescent lamp was filtered through a 5 cm. layer of water to remove the heat and through a Corning No. 3389 filter with a cutoff between 4160 and 4360 Å to remove the long ultraviolet radiations. Each point is the average for twelve individuals and the histograms are for sixteen.

FIGURE 7. Photoreversal of UV injury by visible light. Illustrative experiments: UV dosages, 2475 and 3700 ergs/mm² with wavelength 2554 Å (broken lines) and after photoreactivation of the same with a half hour of visible light applied while the Blepharisma were in a nitrogen atmosphere (solid lines). Controls treated with nitrogen (solid circles) divide like controls kept in air (open circles).
Fig. 6. It is quite clear that visible light alone of the wavelengths transmitted by the filters used is injurious to regeneration of *Blepharisma*. During the course of the experiment, two of the sixteen animals cytolyzed; the others recovered but regeneration was almost 8 hours later than for the control in which regeneration was complete by about 5 hours.

The data in Fig. 6 reveal another fact of importance. While the regeneration is markedly delayed by such weak visible light applied for 12 hours, division of uncut animals is not. Cut *Blepharisma*, both illuminated and not, divide at the same rate as controls. Cut illuminated animals are delayed in starting division because of the retarding effect of visible light upon regeneration which must precede division. It would therefore appear likely that visible light of this intensity affects regeneration and division differentially.

Since division is not unfavorably affected by visible light of this intensity, such light might serve to reverse the injurious effect of UV upon *Blepharisma* as measured by retarded division. Furthermore, since *Blepharisma* subjected to even intense visible light are killed only if oxygen is present (Giese, 1946), removal of oxygen and its substitution with nitrogen might make possible a measurable degree of photoreactivation. Therefore, after irradiation, the *Blepharisma* were placed in a Thunberg tube which was subsequently evacuated. Nitrogen from a tank (99.6 per cent pure), was then substituted for air and the process repeated several times. To test for presence of oxygen a suspension of luminous bacteria was placed in the sidearm of the tube. After each evacuation and replacement the suspension of bacteria was shaken to see whether luminescence could be induced. Luminescence, as seen by the dark-adapted eye, ceases when the oxygen tension falls to about 0.0007 mm. Hg (Harvey, 1940).

Under these conditions photoreactivation was observed, as shown in Fig. 7. Eight series of experiments were performed using dosages from about 2000 to 4400 ergs/mm.² and in each series photoreactivation was obtained. Three series of experiments were also performed in which oxygen was not excluded during photoreactivation. Positive results were also obtained; therefore, oxygen need not be excluded although it would appear that some advantage accrues from its exclusion inasmuch as the photoreactivation was more marked.

**DISCUSSION**

The action spectrum for retardation of regeneration of *Blepharisma* by ultraviolet (UV) light suggests that these radiations affect nucleic acid or nucleoproteins (Hirshfield and Giese, 1953). Since amicronucleate pieces of ciliates will regenerate, provided a piece of the macronucleus is present, whereas amacronucleate pieces do not, even though a micronucleus is present, the macronucleus is thought to control regeneration (see review by Balamuth,
The macronucleus of the strain of *Blepharisma* used in this research undergoes a reorganization during regeneration, condensing and then dispersing again (Suzuki, 1954). Ultraviolet radiation delays macronuclear reorganization. It would, therefore, appear likely that the regeneration-retarding effect of UV is partly, if not mainly, upon the macronucleus.

Soon after cutting, *Blepharisma* is most sensitive to ultraviolet radiations. It is interesting to note that regenerating *Blepharisma* becomes most sensitive to x-rays only 3 hours after cutting (Giese and Lusignan, 1960). These results make it appear likely that UV radiations and x-rays affect different loci of the cell or different processes at the same locus. Since macronuclear reorganization is retarded by both types of radiations, the latter suggestion is more plausible. Lest the correlation between regeneration and macronuclear function be overemphasized it is well to recall that visible light retards regeneration of *Blepharisma*. Since the visible light-sensitive pigment is localized in small granules in the pellicle of the animal, it seems likely that the action of visible light is at least initially confined to this region. Furthermore, failure to obtain photoreversal of UV effects on regeneration is puzzling if the UV effect is only upon the macronucleus, since nuclear effects of UV in general are subject to photoreactivation while cytoplasmic effects are not (Jagger, 1958).

The irradiated regenerating *Blepharisma* appears to recover from UV injury in two steps, one rather rapid, the second slow. This is indicated by the fact that if *Blepharisma* is cut soon after irradiation, regeneration occurs more slowly than if it is cut several hours after irradiation. It may be that UV radiations affect both nucleus (macronucleus) and cytoplasm and that the injury to the latter is more quickly overcome, as in the case of recovery from heat sensitization where very short UV absorbed mainly by the cytoplasm has a transient effect compared to longer UV absorbed selectively by the nucleus (Giese and Crossman, 1945).

The discrepancy between the present observation of distinct photoreactivation of the retardation of division by UV radiations and the failure to achieve this in a previous study in which visible light never photoreactivated and sometimes even injured *Blepharisma* (Hirschfield and Giese, 1953), requires resolution. In the present study when the *Blepharisma* were already excessively damaged by the UV radiations, subsequent illumination proved too much for the animals and harmed them rather than helped them. In general, larger

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1. It is possible that photoreversal of UV retardation of regeneration was not observed because appropriate conditions were not provided. The search for such conditions, if indeed they exist, continues.
2. The previous study was made with *Blepharisma undulans var. americanus*. The cultures of this variety were unsatisfactory at the time the present experiments were started, division being much slower than in past years; therefore a culture of the variety *B. undulans japonicus* was used instead. Since individuals of the variety *japonicus* are much larger than those of *americanus* they are easier to cut and handle.
dosages of UV were used in the previous study than in the present. These results call to mind the studies on photoreactivation by long UV in Colpidium colpoda (Giese et al., 1953). Long UV by itself injured Colpidium when large enough dosages were used, yet long UV in smaller dosages reversed the damage produced by short UV, but only when the damage from short UV was not too great. When Colpidium was already badly injured by short UV, subsequent exposure to long UV proved injurious rather than beneficial.

In the present study negative results were also obtained when, in an attempt to get a higher degree of photoreactivation, the period of illumination was extended from one-half to 1 hour. In a goodly number of trials some photoreactivation was obtained, but in other cases the Blepharisma were damaged and division was retarded even more than in animals treated with UV alone. Since the same lamps had been used in the previous study as in the present, and illumination was continued for at least an hour, it is possible that too much visible light was previously used. This coupled with larger UV dosages previously employed precluded observation of photoreversal.

Most interesting, however, is the finding of good photoreactivation of UV retardation of division when conditions are right. Blepharisma is thus effectively removed from the list of species not subject to photoreactivation. Perhaps more delicate manipulation of the relationships between UV dosage and photoreactivating visible light exposure may lead to evidence for photoreactivation in other refractory species as well (Jagger, 1958).

While photoreversal of UV retardation of division was obtained in Blepharisma, in no case was it as complete as in some of the other protozoans tested, e.g. being of the order of 35 to 40 per cent for Blepharisma while it is 90 per cent for Colpidium (Giese et al., 1952) and Didinium (Brandt et al., 1955). It is conceivable that more favorable conditions than those used here may yet be found for photorecovery in Blepharisma.

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