STUDIES OF CHLOROPLAST DEVELOPMENT IN *EUGLENA*

XV. Factors Influencing the Decay of Photoreactivability of Green Colony Formation

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ABSTRACT When UV-treated cells of *Euglena gracilis* var. *bacillaris* are incubated in the dark in a nutrient medium which permits cell division, they lose the ability to be photoreactivated. The rate of this loss increases with the UV dose. For any given UV dose, the rate of decay increases with increasing growth rate. The same phenomena are observed in light-grown and in dark-grown cells, although the sensitivity to UV of the light-grown cells is smaller by a factor of 1.7. The kinetics of photoreactivation (PR) change during the decay of photoreactivability only if the cells are incubated in growth medium. A UV-inactivation curve for cells photoreactivated only after appreciable PR shows the same slope as that for untreated cells (number of UV-sensitive targets). These results are discussed from the point of view of possible models.

INTRODUCTION

In an earlier paper, we described the loss of ability to be photoreactivated that occurs when ultraviolet- (UV) irradiated cells of *Euglena* are permitted to divide (1). Attempts were made to explain the observed decay curves by a simple dilution model, but none of the available procedures provided a close fit over the entire decay curve. In this paper, we describe experiments extended to a wider interval of UV doses, intended to detect the controlling parameters of decay and to construct a more sophisticated model.

MATERIALS AND METHODS

Euglena gracilis var. *bacillaris*, its growth and conditions of UV irradiation, photoreactivation (PR), and plating have been described previously (1-4). Metal-poor medium is the same as the irradiation medium described by Lyman et al. (4).

For decay experiments, 3.5 ml of a stock culture of *Euglena* were irradiated with UV without dilution, inoculated into a flask of growth medium, and incubated in the dark

at room temperature (about 25°C). At time 0′ and at various times thereafter, aliquots were diluted appropriately and plated. The plates were then incubated in the light. In experiments to study the kinetics of PR during decay, an undiluted suspension of stock culture was treated with UV light, inoculated into growth medium, and incubated in the dark. A time 0′ and at various times thereafter, an aliquot was diluted into "resting medium" (the medium generally used for irradiation experiments) and exposed to PR light.

RESULTS AND DISCUSSION

Decay of Photoreactivability as a Function of UV Dose. Dark-grown cells of *Euglena* were irradiated with various doses of UV light, inoculated into growth medium, and sampled at various stages of growth thereafter, each sample being plated under conditions of PR as described in the Methods section.

Fig. 1 shows that the decay of photoreactivability of green colony formation varies

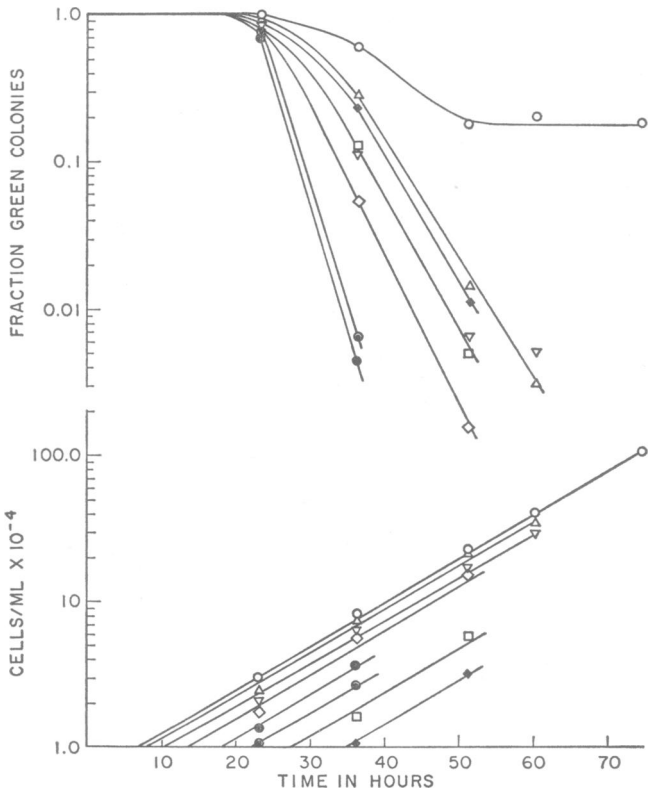

FIGURE 1 The decay of photoreactivability of green colony-forming ability of dark-grown *Euglena*. Irradiated cells were inoculated into growth medium and incubated in the dark. At various times aliquots were withdrawn and incubated in the light. Cells per milliliter were determined by hemocytometer counts and by calculation from the total number of colonies per plate, adjusted for the dilution. Cells per milliliter are adjusted to 1.0×10^4 at time 0′. ○ UV 48, △ 96, ▽ 144, ◇ 192, ● 288, ⊙ 384, □ 576, ◆ 768 ergs/mm².

in a complex way. The lowest dose of UV light used produced the slowest rate of decay during subsequent division of the cells. At this dose, about 20% of the cells produce green colonies, whether they are photoreactivated or not (2). At doses between 48 and 285 ergs/mm^2 there is an increase in the rate of decay with UV dose. At doses above 380 ergs/mm^2, however, there is a decrease in the rate of decay, so that after a dose of 760 ergs/mm^2, the decay is similar to that at 96 ergs/mm^2. The lower curves in Fig. 1 show that the growth rate of the cells is not affected by the UV dose used, but the length of the lag period before division begins increases with increasing UV dose. Consequently, at a dose of 760 ergs/mm^2 there is significant loss of photoreactivability even before the cells begin to divide. Fig. 2 shows the data from Fig. 1 replotted as a function of generation number. In this

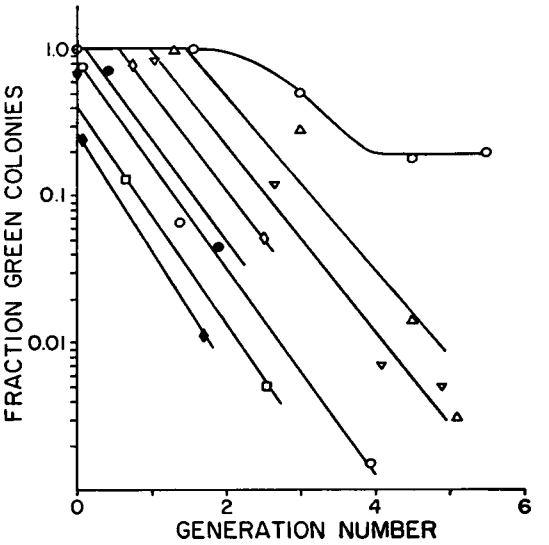

FIGURE 2 The decay data from Fig. 1 are replotted as a function of generation.

case, the decay rates are the same for all UV doses but the very lowest, once the lag has been overcome. The loss of photoreactivability even before growth begins, after high doses of UV, is more obvious in this plot.

To ascertain how decay of photoreactivability during growth is determined by the rate of growth, studies were carried out in which the growth rates were varied by varying the temperature. Cells were adapted to the various temperatures before the experiments. UV irradiation was carried out as above, and growth was permitted to take place at the temperatures to which the cells had been preadapted. Fig. 3 shows decay at 20°C and at 25°C after two different doses of UV light. When the abscissas of the growth curves for the two different temperatures (but same UV dose) are adjusted to make the growth curves coincide, the decay curves also coincide. This shows that the decay rate during growth is determined by the growth rate of the cells.

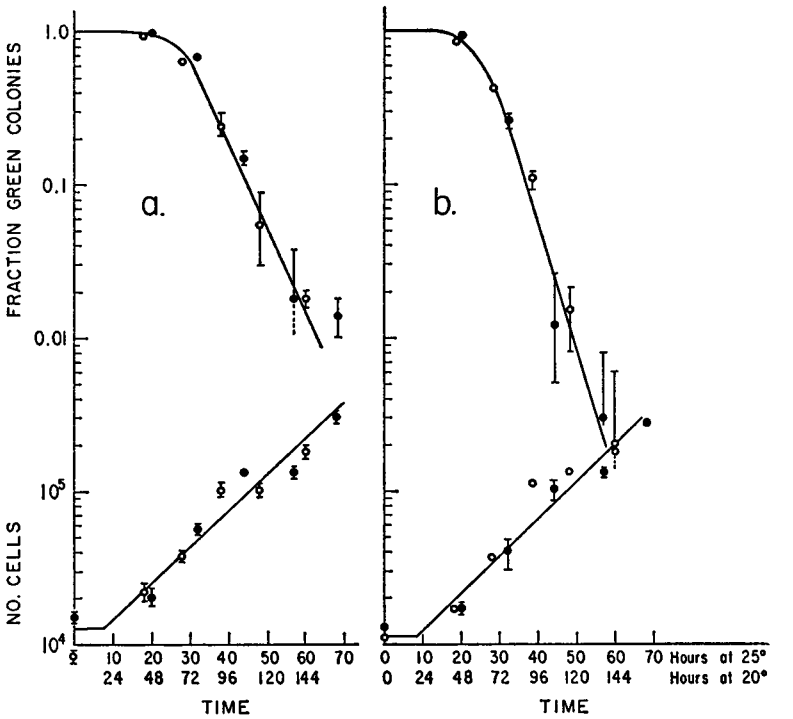

FIGURE 3 The decay of photoreactivability at 20° and at 25°C in dark-grown cells. *a.* A suspension of cells which had previously been growing at 25°C was irradiated (UV 96 ergs/mm²), and treated as in Fig. 1. A second suspension pregrown for 3 days at 20°C was similarly treated. ● Growth and decay at 25°C, ○ growth and decay at 20°C. Spread of the data is indicated. *b.* Same as *a.*, but UV 192 ergs/mm².

Varying the growth rate was also achieved by growing cells on normal and metal-poor medium at the same temperature. Fig. 4 shows that in this case also the decay is determined by the growth rate.

Light-grown cells show the same decay as dark-grown cells under the same conditions (Fig. 5). In the preceding paper (3), it was shown that the rates of PR of dark-grown and light-grown cells are the same for effectively the same UV dose (remembering that dark-grown and light-grown cells differ in sensitivity by a factor of about 1.7). If the kinetics of decay are also the same (for comparable UV doses), it should be possible to superimpose the decay curves if the growth curves are the same. Fig. 6 shows that this is indeed the case. It substantiates the view that we are dealing with the same UV-sensitive entities in the two cases, a conclusion reached previously from other data (2, 4).

Influence of Growth on Kinetics of PR. Dark-grown cells were irradiated with a single UV dose and aliquots were incubated in growth medium in the dark. At various times, samples were removed and the kinetics of PR were measured by

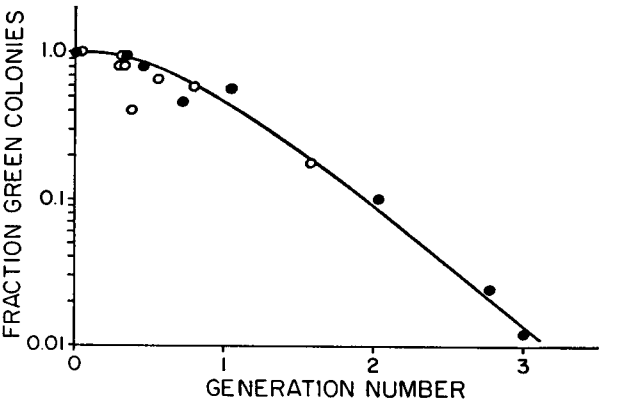

FIGURE 4 The decay of photoreactivability at different growth rates. ● Decay in growth medium, ○ decay in metal-poor medium.

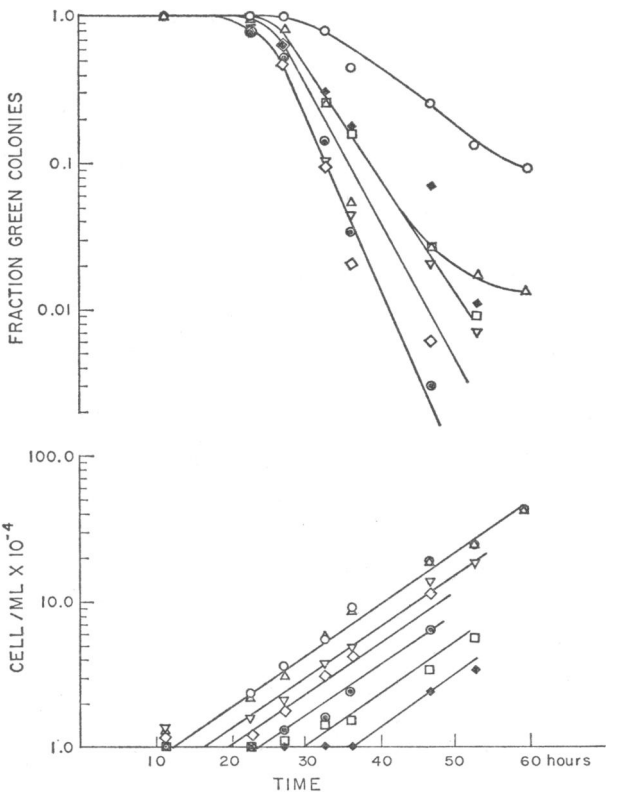

FIGURE 5. The decay of photoreactivability in light-grown cells. ○ UV 48, △ 96, △ 144, ◇ 192, ⊙ 288, □ 384, ◆ 480 ergs/mm².

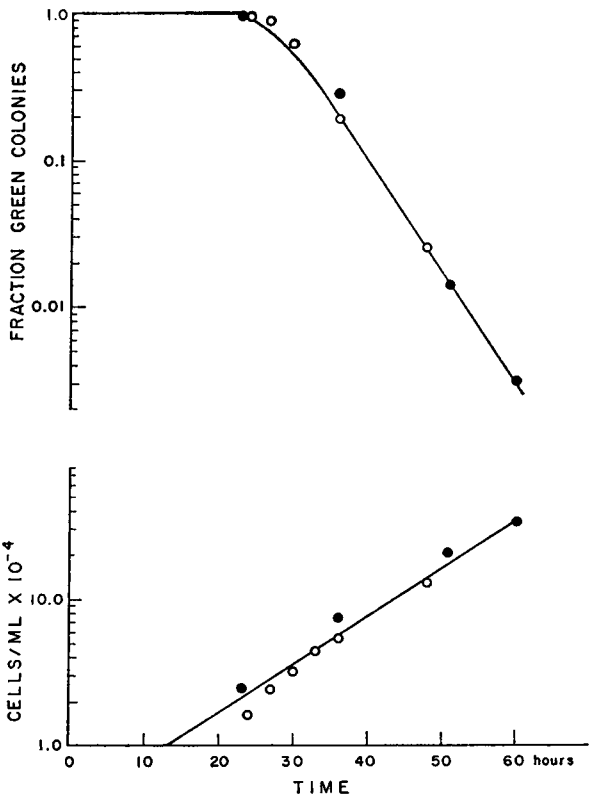

FIGURE 6 A comparison of the decay of photoreactivability in dark-grown and light-grown cells. These curves are from separate experiments. ● Dark-grown cells, UV 96, ○ light-grown cells, UV 160 ergs/mm².

giving various PR doses and plating under nonphotoreactivating conditions. Fig. 7 shows that the kinetics of PR depend on the time spent in growth medium after UV irradiation. The rate of PR decreases with increasing time after UV irradiation. The shapes of the PR curves are also changing with time, so that they cannot be superimposed by adjusting a scale factor. During the time spanned by this experiment (see Fig. 1) there is no significant loss in total photoreactivability, and no increase in cell numbers.

In resting medium, the shape of the PR curves remains essentially the same with time. It was shown previously that cells held under nondividing conditions experienced little or no decay of photoreactivability over long periods of time (1). Although the shapes of the PR curves remain the same, the rate of PR varies somewhat (Fig. 8). The most likely explanation is that the inocula used differed in age. It was shown in a previous paper (3) that the rate of PR varies considerably with the age of the cells.

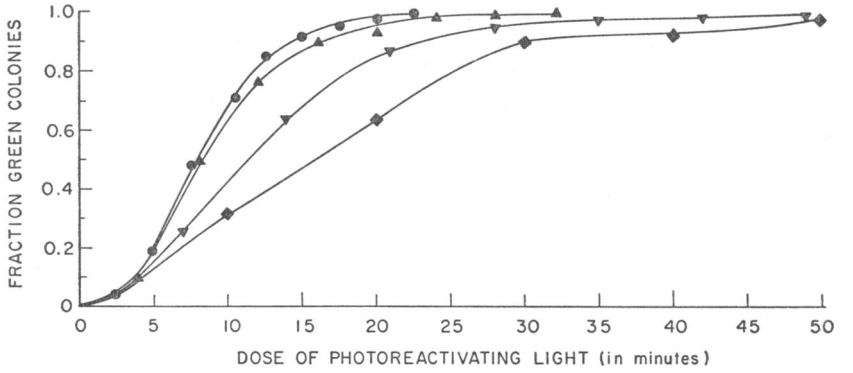

FIGURE 7 The kinetics of PR during the decay of photoreactivability. A suspension of dark-grown *Euglena* was treated with 96 ergs/mm² of UV light, and then inoculated into growth medium. Aliquots were withdrawn at various times thereafter and treated with increments of PR light. ● 0 time after inoculation into growth medium, ▲ 8 hr, ▼ 16 hr, ◆ 24 hr.

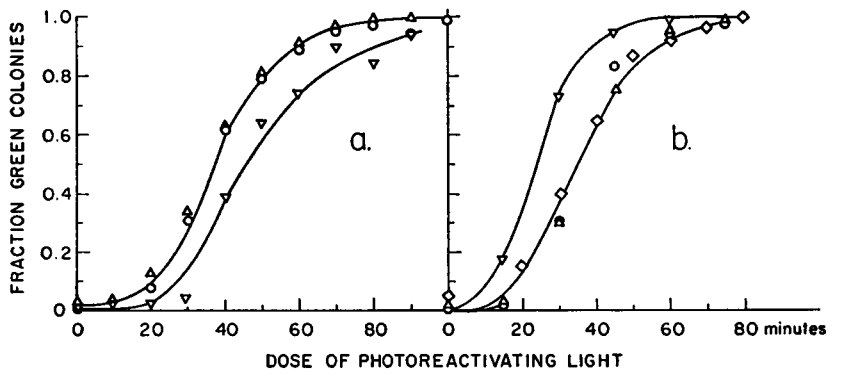

FIGURE 8 The kinetics of photoreactivation in resting medium. *a.* A suspension of light-grown *Euglena* was inoculated into resting medium and incubated in the light for 4 days. Aliquots were irradiated (144 ergs/mm²), held in the dark for various times, and then treated with increments of PR light. ○ PR at 0 time after UV, △ at 4 hr., and ▽ at 12 hr. *b.* Same as *a.*, except that the cells were preincubated in resting medium for 3 days prior to UV. Symbols as for *a.*; ◇ 24 hr.

Kinetics of UV Inactivation after Decay and PR. To determine the nature of the UV-sensitive entities after some decay, dark-grown cells were irradiated with UV light, incubated in growth medium for 24 hr, exposed to PR light for 7 hr on resting medium, and were then exposed to various doses of UV light. The UV-inactivation curves before and after this regime are shown in Fig. 9. Extrapolating the asymptotes back to zero dose gives target number or multiplicity. The multiplicity of the inactivation curve obtained after the treatment is lower than that of the control curve, but the ultimate slopes remain the same. This indicates that all

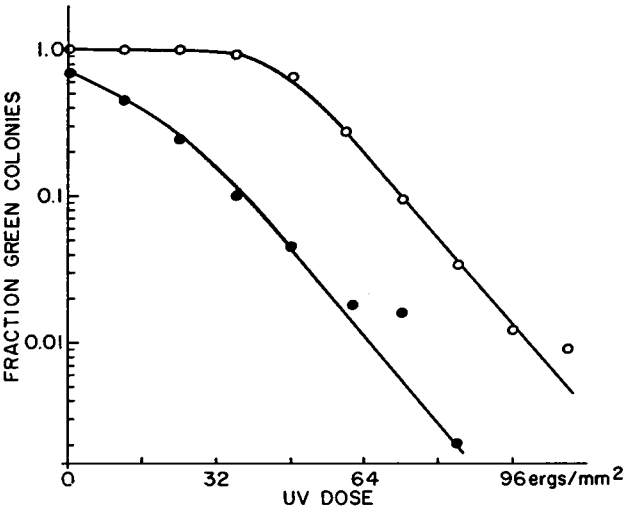

FIGURE 9 The UV inactivation of green colony-forming ability of cells which have been treated with UV light (192 ergs/mm^2), incubated in growth medium for 24 hr, and photoreactivated for 7 hr. ● Cells so treated, ○ unpretreated controls.

entities restored by PR after a period on growth medium have the same sensitivity as the original entities.

CONCLUSIONS

With this paper we have completed a series of studies aimed at elucidating the effect of UV light and of PR in *Euglena*. We will now discuss briefly the results of this paper and then attempt a general discussion of all our results, insofar as they bear on relevant models.

Discussion of Present Results. Analysis of the new data on the decay of photoreactivability confirms earlier assumptions that the decay does not proceed by simple dilution (1). The semilog plots of decay vs. generation time (Fig. 2) reveal that after a lag period, whose length is dependent on the UV dose, the asymptotic rates of decay are about the same for all doses: photoreactivability decays by a factor of 6 during each generation, (whereas a simple dilution model would require a factor of 2).

A factor greater than 2 can have at least two explanations. (*a*) There may be a loss of photoreactivability of the entities accompanying the dilution; (*b*) there may be interactions among entities involved in PR during the dilution process. The first explanation requires that what might be called the intrinsic photoreactivability decreases by a factor of 3 per generation (i.e. by the factor in excess over 2). This possibility will be discussed in a future paper, in which a general model will be

presented. As regards the second explanation, a model for PR decay can be constructed utilizing the multiplicity involved in PR during the dilution process in several ways.

For illustration, let us assume that 2 entities are required for the survival of green colony-forming ability. If these assort independently, then after sufficient dilution one of each type will appear in each photoreactivable cell. From this point on, the average decay will be by a factor of 4 per generation. This still does not account for the observed factor of 6. We have to invoke a loss of "intrinsic photoreactivability" of entities, in addition to a two factor mechanism.

A more promising way of introducing multiplicity stems from other work in this laboratory, which indicates that proplastids associate by threes to form choloroplasts. Let us assume that this association is a necessary one. Then, whenever the ultimate slope of the decay curves is reached, we have a situation in which the photoreactivable cells contain precisely 3 proplastids which can associate. In the next generation, the probability that all 3 will end up in one of the daughter cells will be the product of the individual probabilities $(\frac{1}{2})^3 = 1/8$. If there is any tendency of proplastids to stick together, the dilution decay factor will be less, and this could account for the observed value of 1/6. This kind of explanation is in harmony both with the decay facts and with previous clues about the structures in the chloroplasts. With a model of this type, the decay which takes place in the first generation is, presumably, an expression of the physiological damage by radiation.

Discussion of a General Model. The interpretation of the several phenomena studied in this series presents a serious problem in that the apparent target numbers and number of parameters appear so great that it is, a priori, not very promising to undertake a target type analysis. We, nevertheless, have presented such an analysis for two reasons. First, complications associated with general physiological damage makes such a nontarget analysis very uncertain. Second, three experimental results give strong support to the target interpretation. The interpretation of the UV bleaching curves as revealing 30 targets has been supported by finding that there are, in fact, several dozen proplastids, so that the rather unlikely number of targets turns out to have a firm cytological basis. Our recent results, showing the existence of DNA associated with *Euglena* plastids (6, 7), support the idea that there is DNA in each proplastid, which is conserved upon chloroplast replication.

The second experimental support for the target approach was detailed in the preceding paper in this series (3), in which it was shown that UV inactivation, followed by PR, restores cells completely to their original state so that a subsequent UV bleaching experiment yields a curve identical with the original one, showing that there is no detectable general physiological damage.

The third experimental support for the target approach was given in this paper, in which it was shown that the asymptotic slopes of all PR decay curves are equal.

If there had been appreciable physiological damage, the more heavily irradiated cells should exhibit a faster decay, and there is no such effect for cells receiving UV doses differing by a factor of 20. (There is direct evidence that physiological damage is occurring, as expected, in other experimental circumstances. For instance, the PR doses needed for maximal reactivation do, in fact, increase steadily with increasing UV dose.) This suggests that the decay process does not involve the extent of damage to the photoreactivable structures. The alternative is that we are dealing with the individual structures themselves, and that therefore there are individual structures or targets.

We now present several models which appear promising to us as target models. These models are based on the following facts.

(*a*) All UV-inactivation curves have target numbers of 30 to 40.

(*b*) The target numbers for PR increase steadily, from 1 for the lowest UV inactivating doses, to some finite number >1 for the highest UV doses.

(*c*) The PR decay curves look roughly like those which would be obtained if there were a simple dilution of photoreactivable entities. Quantitatively, however, they do not fit any simple dilution model because (*a*) the number of entities being diluted would have to change with changing dose and (*b*) the asymptotic decay per generation is more than the ½ which would be expected from simple dilution.

The second point introduces the complication that PR *target numbers* greater than unity are obtained. At first sight, it would appear likely that the PR energy would cause rehabilitation of one of the UV damaged entities, thereby restoring the green colony–forming ability of the cell. Finding such a situation for very low doses of UV supports the idea that this is the preferred mechanism. Thus, the greater PR target numbers at higher doses must be interpreted in terms of a more complicated mechanism. One could assume that the individual targets have multiple sites which must be intact to permit the entity to function. Then at UV doses greater than the very lowest doses given, one needs to reactivate more than one site per proplastid. Such a model has been developed by Hill and Alling (8) and is presented in detail in a separate paper by them. On the other hand, one might assume that there are interactions among proplastids, that can restore all functions of an individual plastid or target. This is a kind of multiplicity reactivation, similar to that found in bacteriophages. At higher doses, it might be more feasible to restore a few functions in each of several UV-damage plastids than to rehabilitate an entire single plastid.

The PR decay curves have been interpreted above as being consistent with the view that the association of several proplastids occurs in the construction of a chloroplast. A combination of the proplastid association scheme and a two factor scheme would also serve for a quantitative explanation of the decay data. The fact that the action spectrum for the UV inactivation shows peaks at both 260 and 280 $m\mu$ (4) suggests that 2 factors may be involved; these are peaks for nucleic acids and for proteins. In any event there seems to be a requirement for cooperative ac-

tivity among proplastids, a feature already invoked to explain the PR curves themselves.

In summary, then, there are several possible ways of understanding the photobiological facts. The simplest composite model is one in which there are about 3 dozen nucleoprotein-containing proplastids, independently capable of giving rise to green, replicating chloroplasts, provided there are at least 3 of them. These chloroplasts can cooperate, in adversity, to retain the green colony–forming ability they have individually lost.

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