* This investigation is aided by the U.S. Public Health Service research grant no. AI-3405-03 and the research career development award 5-K3-GM-15, 434-04. The author is indebted to Dr. Armin Braun of the Rockefeller Institute for supplying the tobacco plants used in this investigation.

[1] Abbreviations: TMV, tobacco mosaic virus; TMV-RNA, tobacco mosaic virus ribonucleic acid; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetate; TCA, trichloroacetic acid; RNAase, ribonuclease.

[2] Reddi, K. K., and Y. V. Anjaneyalu, *Biochim. et Biophys. Acta*, **72**, 33 (1963).
[3] Reddi, K. K., these PROCEEDINGS, **50**, 75 (1963).
[4] Markham, R., and J. D. Smith, *Biochem. J.*, **52**, 558 (1952).
[5] Reddi, K. K., *Biochim. et Biophys. Acta*, **27**, 1 (1958).
[6] Sugiyama, T., and H. Fraenkel-Conrat, *Biochemistry*, **2**, 332 (1963).
[7] Staehelin, M., *Biochim. et Biophys. Acta*, **43**, 29 (1959).
[8] Privat de Garilhe, M., and M. Laskowski, Sr., *J. Biol. Chem.*, **223**, 661 (1956).
[9] Razzel, W. E., and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 1770 (1958).
[10] Frisch-Niggemeyer, W., and K. K. Reddi, *Biochim. et Biophys. Acta*, **26**, 40 (1957).
[11] Reddi, K. K., *Biochim. et Biophys. Acta*, **28**, 386 (1958).
[12] Reddi, K. K., *Biochim. et Biophys. Acta*, **33**, 164 (1959).
[13] Reddi, K. K., unpublished data.
[14] Reddi, K. K., *Biochim. et Biophys. Acta*, **36**, 132 (1959).

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*THE EFFECT OF ACRIFLAVINE ON PHOTOREVERSAL OF LETHAL AND MUTAGENIC DAMAGE PRODUCED IN BACTERIA BY ULTRAVIOLET LIGHT**

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Communicated by M. Demerec, July 25, 1963

Mutations to prototrophy, induced in auxotrophic bacteria by far ultraviolet light (UV), may be eliminated by subsequent exposure to near UV or visible light (photoreversal),[1] or, in the dark, by temporary suppression of postirradiation protein synthesis ("dark repair").[2] It has been proposed[3] that photoreversal and "dark repair" of these mutations involve alternative mechanisms for the enzymatic repair of the same premutational damage produced by UV in the bacterial deoxyribonucleic acid (DNA). Since killing by UV is photoreversible, but not subject to "dark repair" (in *Escherichia coli* B/r), and since the same is true of certain UV-induced mutations other than those to prototrophy,[4] it seems certain that UV produces at least two kinds of photoreversible lesions in bacteria, only one of which is also subject to "dark repair."

Acriflavine has been shown to interfere with "dark repair" of UV-induced prototrophy, and it has been suggested that it does so by combining with and modifying the irradiated bacterial DNA in a way that reduces the accessibility of the premutational lesions to the postulated "dark repair" enzyme.[5] If the lesions leading to prototrophy are indeed different from those leading to death, acriflavine might be expected to interfere also with photoreversal of these mutations, but not necessarily with photoreversal of killing. In this report, experiments are described in which the effect of acriflavine on photoreversal of both lethal and mutagenic

damage initiated by UV was determined. Similar experiments in which illumination with visible light preceded exposure to UV (photoprotection)[6, 7] are also described.

Material and Methods.—A tyrosine-requiring derivative of *Escherichia coli* B/r, strain WU36, was used. Cultures were grown overnight, on a shaker, in Difco nutrient broth, with 0.5% NaCl added. One-ml aliquots were diluted in nine ml of fresh nutrient broth, incubated for 45 min, then chilled for ten min in the refrigerator. The bacteria were then washed and resuspended in saline solution (0.9% NaCl) at a titer of about 10^8 cells per ml.

The source of UV was a General Electric germicidal lamp, emitting primarily light of 2537 Å, and having an intensity of 6.6 ergs per second per mm^2 at a distance of 60 cm. The saline suspensions of bacteria were exposed to UV in open Petri dishes, with agitation during the exposure. Yellow light was used to illuminate all subsequent operations, except photoreversal.

The source of photoprotecting or photoreversing light (PPL or PRL) was a 650-watt photoflood bulb in a Westinghouse Studio One camera light. Pre- and post-treatment with visible light was administered just before or after irradiation with UV. The saline suspensions of bacteria were placed in test tubes, which were immersed in a bath of running cold tap water (14°C), at a distance of 3 cm from the lamp. Under these conditions, maximum photoreversal of both killing and induced prototrophy was obtained in strain WU36 after five min of exposure to PRL. "Dark repair" of induced prototrophy does not occur under these conditions, as shown by controls not included here, and can be ignored. Dark controls were treated exactly like the illuminated samples, except that the tubes were wrapped in opaque aluminum foil.

Solid media consisted of minimal "E" medium[8] with 0.4% glucose, supplemented with 5% (by liquid volume) nutrient broth (SEM agar), and SEM to which acriflavine was added, to give a final concentration of 0.0001%, just before pouring the plates (SEM-AC agar). This concentration of acriflavine has no measurable effect on survival or rate of mutation to prototrophy in unirradiated WU36. The bacteria were plated immediately after exposure to PRL in photoreversal experiments, and immediately after irradiation with UV in photoprotection experiments. All incubations were for two days at 37°C. Assays for survival were always performed on the same medium used to determine mutation frequency. Neutral acriflavine was obtained from Nutritional Biochemicals Corporation.

Results.—Figure 1 shows the effects of acriflavine on survival, as a function of UV dose, in the dark, and when visible light is administered before and after exposure to UV. Examining first the dark survival curves, it will be seen that the addition of acriflavine to the postirradiation plating medium markedly decreases UV survival, changing the form of the survival curve to the "one-hit," two-slope type usually obtained with the sensitive B strain. When visible light is administered after UV (PRL curves), the expected photoreversal characteristic of B/r[9] is obtained on SEM plates. About the same amount of photoreversal is obtained on SEM-AC plates, as manifested by the similarity of the "dose reduction" obtained on both kinds of plates. Thus, acriflavine does not significantly interfere with photoreversal of UV killing. The PPL curves show that a small but definite photoprotection against UV killing results from pre-UV exposure to visible light,

whether plating is on SEM or SEM-AC agar. This finding contradicts reports that *E. coli* B/r is not photoprotectible against UV killing.[10, 11] (The wild type B/r strain from which WU36 was derived was found to be equally photoprotectible.) The magnitude of maximal photoprotection is small compared to that of maximal photoreversal, and requires about four times as much visible light. The presence of acriflavine in the postirradiation plating medium does not affect the amount of photoprotection accomplished, as indicated by the similarity of the "dose reduction" on SEM and SEM-AC plates. Acriflavine therefore does not interfere with photoprotection against UV killing.

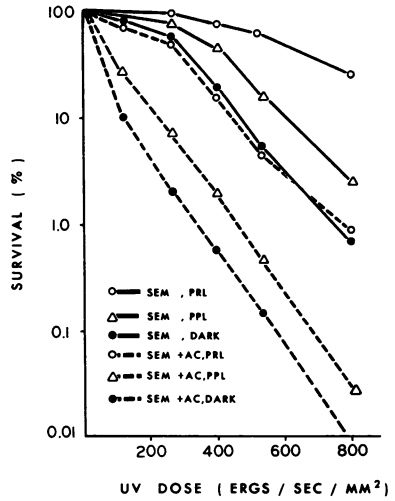

FIG. 1.—Effect of acriflavine on UV survival. Titer at 100% survival = 1.2×10^8 bacteria per ml; PRL—six min of visible light after UV; PPL—20 min of visible light before UV. Each point is the average of three similar experiments.

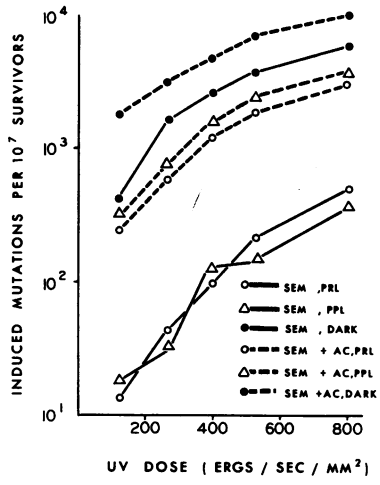

FIG. 2.—Effect of acriflavine on frequency of UV-induced prototrophs. PRL—six min of visible light after UV; PPL—20 min of visible light before UV. Each point is the average of the same three experiments used to determine survival as shown in Fig. 1.

Figure 2 shows the frequency of UV-induced prototrophs, as a function of UV dose, under the same conditions shown in Figure 1 for survival. Examining first the dark controls, it will be seen that somewhat higher yields of induced mutations are obtained on plates containing acriflavine. This "enhancement" effect has been described in an earlier report,[5] and has been interpreted as the consequence of the prevention, by the dye, of residual "dark repair" that normally occurs even on enriched media. The PRL curves show the expected photoreversal of tyr+ mutations on SEM, as described previously.[3] Much less photoreversal of mutation is obtained, however, when plating is on SEM-AC. Acriflavine appears to interfere with photoreversal of these mutations, as well as with their loss by "dark repair." The PPL curves show that pre-UV illumination with visible light reduces the yield of UV-induced prototrophs just as effectively as post-UV illumination with PRL. Photoprotection against induced prototrophy is much more effective than photoprotection against killing, with the same exposure to PPL. When plating is on SEM-AC, relatively little photoprotection against induced prototrophy occurs.

Acriflavine thus interferes with photoprotection against induced prototrophy, as well as with photoreversal and "dark repair" of these mutations.

There are two possible ways of interpreting the smaller degree of photoreversal and photoprotection obtained for induced mutations on SEM-AC plates. The acriflavine may act by affecting the intrinsic reversibility of the premutational lesions leading to induced prototrophy, or it may lower the efficiency of repair so that the amount of visible light used is no longer sufficient to permit maximal repair. To distinguish between these possibilities, experiments were done in which (at a single dose of UV) the time of exposure to PPL or PRL was varied 0–25 min. Results for PRL are shown in Figure 3.

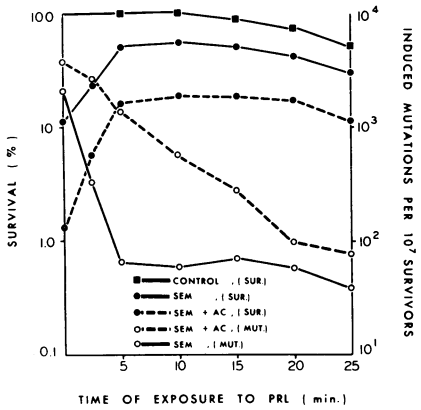

FIG. 3.—Photoreversal of killing and induced prototrophy at various doses of PRL. UV dose—600 ergs per mm;^{2} titer at 100% survival = 1.4×10^8 bacteria per ml. Each point is the average of three similar experiments.

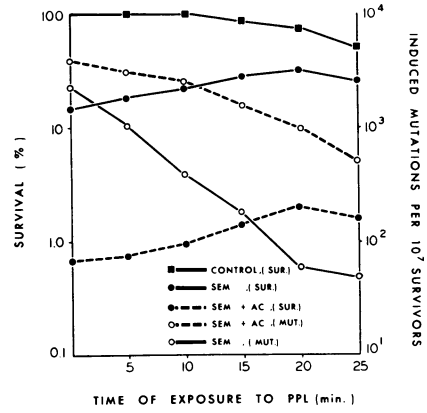

FIG. 4.—Photoprotection against killing and induced prototrophy at various doses of PPL. UV dose—600 ergs per mm;^{2} titer at 100% survival = 1.4×10^8 bacteria per ml. Each point is the average of three similar experiments.

The control curve shows the effect of increasing time of exposure to PRL on unirradiated bacteria. Some lethality is observed, starting at about 15 min of exposure. Maximal photoreversal of killing is obtained, on both SEM and SEM-AC plates, with exposures of five min or longer, and the same is true for photoreversal of induced prototrophy when plating is on SEM. When acriflavine is present in the postirradiation plating medium, however, maximal photoreversal of induced prototrophy requires about 20 min of exposure to PRL, as compared with the five min required to accomplish reversal when plating is on SEM. Acriflavine therefore does not alter the *photoreversibility* of the potential mutations, but markedly reduces the *efficiency of the photoreversal.*

A comparable experiment, in which visible light is administered before UV, is shown in Figure 4. Maximal photoprotection against killing requires about 20 min of exposure to PPL, when plating is on SEM or SEM-AC, as does maximal photoprotection against induced prototrophy when plating is on SEM. When plating is on SEM-AC, however, photoprotection against induced prototrophy is still far from the maximal level after 25 min of exposure to PPL. Exposures longer than this are impractical because of lethal effects of PPL itself. It is therefore not possible to determine whether photoprotection would continue, with longer

exposures to PPL, until the achievement of maximal protection. After 20 min of PPL, the same amount of photoprotection is achieved on SEM-AC plates as after five min of PPL on SEM plates. It is clear that the presence of acriflavine in the postirradiation plating medium reduces the efficiency of photoprotection against induced prototrophy.

Discussion.—Differential responses of lethality and mutagenesis to PRL after UV have been noted by Kaplan and Kaplan,[12] working with s-mutations in *Serratia*, from which they concluded that the processes leading to inactivation and mutation are not the same. In other studies,[9, 13, 14] the similarities between photoreversal of the lethal effects of UV and that of the particular mutational systems studied have been more impressive than the differences. The fact that different bacterial mutation systems react differently to UV and PRL, and may originate in different initial steps, was emphasized by Zelle *et al.*[14] There can be little doubt, on the basis of the differential effects of acriflavine, that UV-induced mutations to prototrophy originate through changes which differ from those leading to photoreversible killing.

Considerable recent evidence indicates that photoreversal involves enzymatic repair of UV-produced damage in DNA. A "photoreactivating enzyme," isolated from *E. coli* and yeast,[15] has been shown (when activated by PRL) to mediate the monomerization of dimers of thymine produced by UV in transforming DNA.[16] A mutant strain of *E. coli* B unable to photoreverse UV killing[17] fails to yield "photoreactivating enzyme,"[18] which is readily obtainable from the photoreactivable parent strain. This strongly suggests (unless one assumes more than one specific activity for this enzyme) that most, if not all, of the photoreversible killing in *E. coli* is the consequence of dimerization of thymine. This possibility is consistent with the finding[19] that thymine dimers account for about half the lethal effect of high doses of UV.

Acriflavine interferes with photoreversal of induced prototrophy, but not with photoreversal of killing. Assuming both kinds of photoreversal to be enzymatic, and further assuming that photoreversible killing is largely initiated by dimerization of thymine, there are two possible interpretations of this result. The simpler would be that two distinct enzymes are involved, each acting on a different primary lesion. In this case, it would follow that mutations to prototrophy do not originate through thymine dimers, but through one or more other kinds of UV-initiated lesion in DNA.

It is also possible, however, that the same "photoreactivating enzyme" is involved in the repair of lesions responsible for photoreversible killing and induced prototrophy. If this is so, one must suppose that the initial lesions leading to death and to prototrophy are the same (presumably thymine dimers), and that the differentiating effect of acriflavine depends upon a secondary difference. For example, one might imagine that thymine dimers cross-linking the two strands of DNA (if they occur) and those involving neighboring bases in the same strand could be monomerized by the same enzyme, yet they might be differentially affected by the combination of acriflavine with the DNA. This, or some other secondary difference in spatial organization could result in differential availability of prelethal and preprototrophic lesions for repair by the same enzyme. The fact that induced prototrophy is subject to "dark repair," while UV killing is not, raises the same question. This difference, too, could be explained by assuming that different primary lesions

are involved, or that the difference is in the accessibility of the lesions to the reparative action of the postulated "dark repair" enzyme. Decisive answers are being sought in studies, now in progress, of mutant strains lacking one or another of the repair enzymes.

Summary.—The addition of acriflavine to the postirradiation plating medium has the following effects in strain WU36, a tyrosine-requiring substrain of *E. coli* B/r: (1) it "enhances" both the lethal and mutagenic effects of UV, decreasing survival and increasing the yield of tyr+ mutations; (2) it reduces the efficiency of photoreversal of induced prototrophs, increasing the amount of light required to accomplish maximal photoreversal about fourfold, while having no such effect on the photoreversal of UV killing; (3) it reduces the efficiency of photoprotection against induced prototrophy, while having no such effect on photoprotection against UV killing. Mutations to prototrophy are reducible in frequency by pretreatment with visible light to the same extent as by posttreatment with visible light, although maximal photoprotection requires about four times as much light as maximal photoreversal. In contrast, very little photoprotection against UV killing is obtained in this strain. The results are interpreted as evidence that the lesions leading to photoreversible UV killing and those leading to photoreversible UV-induced prototrophy are different, either in their primary nature or in some unknown secondary feature.

These studies were carried out with the able technical assistance of Mr. Nicholas A. Sicurella.

* This research was supported by grant No. AI-01240 from the National Institute of Allergy and Infectious Diseases of the U. S. Public Health Service.

[1] Jaggar, J., in *Radiation Protection and Recovery*, ed A. Hollaender (New York: Pergamon Press, 1960), p. 352.

[2] Witkin, E. M., in *Genetic Mechanisms: Structure and Function*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 21 (1956), p. 123.

[3] Witkin, E. M., in *Repair from Genetic Radiation Damage*, ed. F. H. Sobels (Oxford, England: Pergamon Press, Ltd., 1963), p. 151.

[4] Witkin, E. M., and E. C. Thiel, these PROCEEDINGS, **46**, 226 (1960).

[5] Witkin, E. M., *J. Cell. Comp. Physiol.*, **58**, Supplement 1, 135 (1961).

[6] Weatherwax, R. S., *J. Bacteriol.*, **72**, 124 (1956).

[7] Jagger, J., *Radiation Research*, **13**, 521 (1960).

[8] Vogel, H. J., and D. M. Bonner, *J. Biol. Chem.*, **218**, 97 (1956).

[9] Novick, A., and L. Szilard, these PROCEEDINGS, **35**, 591 (1949).

[10] Kelner, A., *J. Bacteriol.*, **48**, 511 (1949).

[11] Miki, K., *Japanese J. Bacteriol.*, **11**, 803 (1956).

[12] Kaplan, R. W., and C. Kaplan, *Exptl. Cell Research*, **11**, 378 (1956).

[13] Newcombe, H. B., and H. A. Whitehead, *J. Bacteriol.*, **61**, 243 (1951).

[14] Zelle, M. R., J. E. Ogg, and A. Hollaender, *J. Bacteriol.*, **75**, 190 (1958).

[15] Rupert, C. S., *J. Gen. Physiol.*, **43**, 573 (1960).

[16] Wulff, D. L., and C. H. Rupert, *Biochem. Biophys. Res. Comm.*, **7**, 237 (1962).

[17] Harm, W., and B. Hillebrandt, in *Photochemistry and Photobiology* (Oxford, England: Pergamon Press, Ltd., 1962), vol. 1, p. 272.

[18] Rupert, C. S., personal communication.

[19] Setlow, R. B., and J. K. Setlow, these PROCEEDINGS, **48**, 1250 (1962).