RECOVERY OF COLONY-FORMING ABILITY AND GENETIC MARKER ACTIVITY BY UV-DAMAGED *HEMOPHILUS INFLUENZAE*

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ABSTRACT The rate of recovery of UV-irradiated *Hemophilus influenzae* from acriflavine-sensitized loss of colony-forming ability was studied at various acriflavine concentrations, UV doses, and temperatures. This rate (as calculated from an equation based upon certain assumptions) was on the order of 0.07 per minute per cell at 37°C. This did not vary greatly with UV dose or acriflavine concentration, but did with temperature, giving a $\Delta H\ddagger$ of about 16 kcal/mole. In another set of experiments, cells bearing two genetic markers (resistance to 2000 μg/ml streptomycin and to 2.5 μg/ml novobiocin) were irradiated and then incubated without acriflavine. DNA extracts made from samples taken after various periods of incubation time were assayed on antibiotic-sensitive cells using acriflavine to inhibit repair during and following transformation. It was found that both in vivo irradiated markers were reactivated in the donor to approximately the same extent (with a rate constant of 0.04 per minute). This result was in contrast to the results obtained when extracted DNA bearing the same markers was irradiated in vitro and used to transform cells. In this latter case the streptomycin marker was much more sensitive than the novobiocin marker. This difference is interpreted as being due to the mechanics of the transformation system.

INTRODUCTION

The presence of acriflavine in postirradiation assay media is known to sensitize bacteria (1–5), phage (4–9), and transforming DNA (4) to inactivation by short wavelength (254 mμ) ultraviolet light (UV). Although its mechanism of action is not entirely understood, acriflavine is known to inhibit dimer excision from the DNA of UV-irradiated *Escherichia coli* B and B/r (10, 11). The excision process is found in the relatively UV-resistant *E. coli* strains B, B/r, and AB1157, but not in the *E. coli* UV-sensitive mutants B_{s-1} and AB1886 (12, 13). These facts suggest that acriflavine may sensitize the UV-inactivation of bacteria, phage, and transforming

DNA by stopping a repair process, one step of which is the excision of photoproducts from the DNA of the irradiated entity. Excision has been found to occur also in *Hemophilus influenzae* (14).

This paper describes experiments done to follow the time course of recovery of *Hemophilus influenzae* cells and of their transforming DNA from damage by ultraviolet light. Use was made of acriflavine to block recovery after various intervals of incubation.

MATERIALS AND METHODS

Bacterial Strains

We have used *H. influenzae* strain Rd obtained from Dr. Roger Herriott. This strain has been transformed in our laboratory to 2000 μg/ml streptomycin resistance (Rd Str), and to both 2000 μg/ml streptomycin and 2.5 μg/ml novobiocin resistances (Rd Str Nb) for use in the experiments reported here.

Media

The growth medium consisted of 3.7% Brain Heart Infusion (Difco Laboratories Inc., Detroit, Mich.) supplemented with 2 μg/ml nicotinamide adenine dinucleotide (NAD, Nutritional Biochemicals Corp., Cleveland, Ohio) and 10 μg/ml hemin (Nutritional Biochemicals) unless otherwise specified. This is referred to as BHHN medium. For plating, 1.25% Bacto-Agar (Difco) was added. Nutritionally unsupplemented Brain Heart broth is referred to as BH medium.

Competent cells were prepared in BHHN by the method of Cameron as reported by Barnhart and Herriott (15). To store cells, 15% glycerine was added and the culture was frozen at −65°C. There were about 10^9 cells per ml in the stock cultures.

For some experiments acriflavine (acriflavine hydrochloride, Allied Chemical Corp., New York) was added to the unsupplemented broth (BHA) during transformation and/or to the nutritionally supplemented agar (BHHNA). In these cases plating was done under yellow light to stop photodynamic effects. During the experiments it was found that acriflavine formed a complex with hemin, which reduced the ability of acriflavine to decrease recovery from UV damage. It was thus necessary to be accurate in both hemin and acriflavine concentrations.

Antibiotics

The following antibiotics were used: streptomycin (Streptomycin Sulfate, USP., Eli Lilly and Co., Indianapolis, Ind., Ampoule No. 431) and novobiocin (Cathomycin Sodium, N.F. Merck, Sharp & Dohme, West Point, Pa., Capsule No. 3217 X).

Irradiation

Ultraviolet irradiation of cells was carried out under General Electric germicidal lamps (General Electric Co., Schenectady, N. Y.). For low doses a dose rate corrected for absorbance and scattering of 0.63 ergs/mm²/sec was used. For higher doses a corrected rate of 13 ergs/mm²/sec was used. Cells (10^9/ml) to be irradiated were thawed at room temperature, washed twice in equal volumes of an ice cold buffer consisting of 0.1 M NaCl, 0.01 M Tris, 0.01 M $CaCl_2$, 0.005 M $MgCl_2$, and 0.02% Tween 80 (Nutritional Biochemicals Corp., Cleveland, Ohio) pH 6.9 (TCNT), and irradiated in a Petri dish with agitation while still cold in the same buffer,

General Procedure for Postirradiation Incubation Studies

For observing the time course of recovery from acriflavine sensitization of colony-forming ability, 0.1 ml of an irradiated culture of competent Rd Str Nb cells was pipetted into 2.9 ml of BHHN broth which had been previously equilibrated to a given temperature. Samples of this culture were taken at various times, diluted appropriately in BHA broth, and plated immediately in duplicate in 10 ml of both BHHN and BHHNA agars. The colony-forming ability of samples diluted in BHA broth and then plated on BHHN agar was found to be the same as that when the dilution was done in BH broth.

For observing recovery of transforming ability of DNA extracted from irradiated cells, an irradiated culture of Rd Str Nb cells was centrifuged 2 min at 12,000 *g* and resuspended in an equal volume of 35°C BHHN broth. As a function of time, 0.1 ml samples of this culture were pipetted into 0.9 ml of a lysis buffer consisting of 1.0 M NaCl, 0.01 M ethylenediaminetetraacetic acid (EDTA), and 0.2% sodium lauryl sulfate, pH 8.5. After heating to 60°C for 10 min, the lysates were diluted 1:10 in BH broth. To assay for transformants in the presence and absence of acriflavine, 0.1 ml was pipetted from each dilution into 2.8 ml of both BH and BHA broth. Competent cells (0.1 ml) were then added to each tube and 30 min of incubation at 35°C were allowed for uptake of DNA. Plating was then done in 10 ml BHHN or BHHNA agar. After time for expression was allowed, 10 ml of the appropriate agar plus antibiotic (500 μg/ml streptomycin or 2.5 μg/ml cathomycin) were overlaid onto the plates to select for transformants. During these experiments it was found that acriflavine (0.4 μg/ml) increased the expression time of the streptomycin marker from 2 to 3 hr and of the Nb marker from 1.5 to 3 hr. Therefore, when acriflavine was used, at least 3 hr of expression time were allowed before overlaying.

RESULTS

Survival of Cells

It was found that 0.1 to 0.5 μg acriflavine per ml did not kill nonirradiated cells, but did depress the survival of irradiated cells. By obtaining survival curves of washed competent Rd Str Nb cells plated with and without 0.4 μg acriflavine per ml it was found that the D_{37} was decreased from 60 to 1.3 ergs/mm² by using this agent.

If it is assumed that acriflavine blocks a repair process in *H. influenzae* as has been shown for *E. coli* (10, 11), then allowing an irradiated population time to repair should decrease the sensitivity of the cells to acriflavine. Washed Rd Str Nb competent cells were irradiated with 13 ergs/mm² and subsequently allowed to incubate at 37°C in BHHN broth for various times before plating in BHHN agars containing 0.4, 0.3, 0.2, and 0.0 μg acriflavine per ml. The results in Fig. 1 were obtained. The irradiated population plated without acriflavine had 80% of the nonirradiated colony-forming ability. It was evident that acriflavine increased the UV sensitivity of the cells in a manner dependent upon the concentration of the dye, and also that the cells were able to recover almost completely from the sensitization after 80 min of post-irradiation incubation in BHHN broth at 37°C. When the ratio of the number of UV survivors obtained in the presence of acriflavine (S_a) to the number obtained without acriflavine (S) was taken, the fraction of the UV survivors surviving in the presence of acriflavine at any time (S_a/S) was found. The values for

S_a/S, normalized to a maximum of 80% survival at the maximum level of recovery are shown along with survival curves done at the same acriflavine concentrations in Fig. 2.

It was found that the time course of the increase in S_a/S could be described by an equation that was obtained by following the outline of Roberts and Aldous (16). A "hit" in our analysis is defined as a UV-produced potentially lethal lesion, the repair of which is blocked in the presence of a given concentration of acriflavine, but which is repairable when no acriflavine is present. The assumptions are made that the cells are doublets (microscopically at least 90% of the cells are in doublet

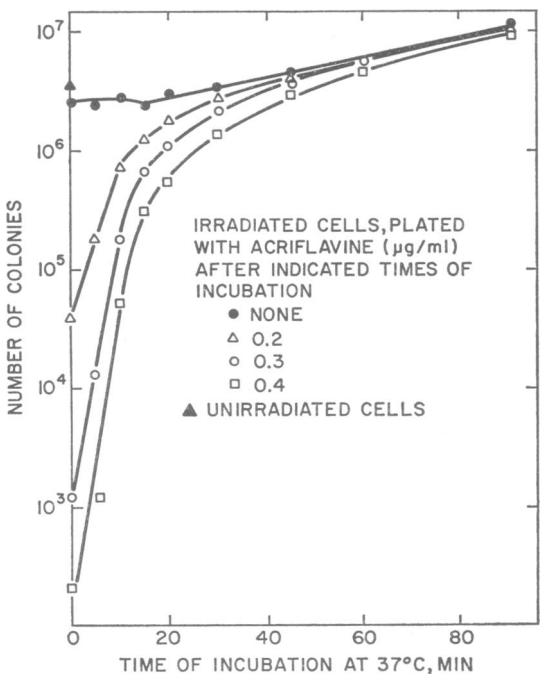

FIGURE 1 Recovery of colony-forming ability of UV-irradiated Rd Str Nb cells as a function of time of postirradiation incubation before exposure to acriflavine.

form) so that "two target" kinetics may be used, that a dose of ultraviolet light produces an average number x of hits per cell in the doublet, that the fraction of hits remaining in a cell after a time t of incubation decreases as e^{-kt} (where k is a first-order rate constant), and that a cell is reactivated (i.e. is an acriflavine-insensitive colony former) when all hits in it are gone. To calculate the fraction S_a/S, the expression for the probability of a doublet having at least one colony-forming unit at any time is first determined. Because there is a distribution of hits per cell in the population, this first expression must then be multiplied by the product of two Poisson distributions (to take into account the fractional contribution to the reactivated population of doublets initially having n_i hits in one cell and n_j in the other) and summed over all hits:

$$\sum_{n_i=0}^{\infty} \sum_{n_j=0}^{\infty} \frac{[e^{-2x} x^{(n_i+n_j)}]\,[1-(1-(1-e^{-kt})^{n_j})\,(1-(1-e^{-kt})^{n_i})]}{n_i\,!\,n_j\,!}$$

The result is

$$S_a/S = 2e^{-xe^{-kt}} - e^{-2xe^{-kt}}. \tag{1}$$

When one considers the same problem under conditions where the cells are singlets (the case of Roberts and Aldous (16)) the result is

$$S_a/S = e^{-xe^{-kt}}. \tag{2}$$

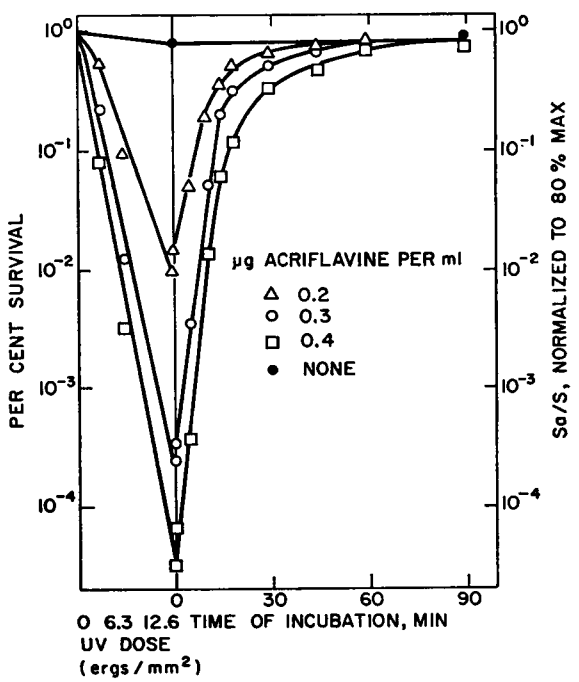

FIGURE 2 Inactivation and recovery of Rd Str Nb cells from acriflavine sensitivity as a function of time of postirradiation incubation in broth containing different concentrations of acriflavine.

It should be pointed out that the recovery process is not necessarily first order and that Michaelis-Menten kinetics might be a better approximation. This latter approach, however, introduces additional variables into the equation, the values of which are difficult to determine in an in vivo system.

When the first-order analysis is applied to the curves in Fig. 2, rate constants of 0.077 per min for 0.2, 0.084 for 0.3, and 0.081 for 0.4 μg acriflavine per ml were found. This relative invariability of the rate constant would be expected in cases where only the number of hits (as defined above) was varied.

To vary the number of hits in another way, similar experiments were done at various UV doses using the same concentration of acriflavine (0.3 μg/ml). In a series of experiments, competent Rd Str Nb cells were washed twice and irradiated for various times to obtain survival curves. A 0.1 ml sample from one of the irradiated cultures was taken for postirradiation incubation at 35°C. In Fig. 3, the

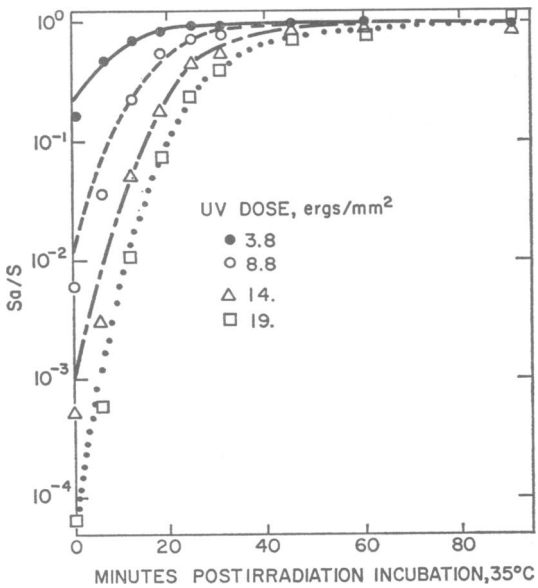

FIGURE 3 S_a/S (the fraction of UV survivors also surviving in agar containing acriflavine) at different UV doses as a function of time of postirradiation incubation. (Points experimental, lines theoretical.)

TABLE I
VALUES FOR AVERAGE NUMBER OF HITS AND RATE CONSTANTS AT FOUR UV DOSES (254 mμ)

UV dose	Average number hits (x)	Rate constant (k)
ergs/mm²		*min⁻¹*
3.7	2.1	0.082
8.5	5.0	0.074
13	7.9	0.067
19	10.7	0.065

experimental points are shown along with the theoretical curves, which were made by obtaining the value for x from the average of all the survival curves and adjusting k for the best fit of the data. The value of x was obtained by fitting the survival curves to the equation for a two-target survival curve $[N/N_0 = 1 - (1 - e^{-x})^2]$. A linear relationship was obtained with a slope of 0.59 hits per erg/mm². The UV doses and values for x and k, are given in Table I. Similar results were obtained in another such series of experiments.

Dependence of the Rate Constant upon Temperature

The repair rate was further characterized with respect to its dependence on temperature by holding the postirradiation culture in BH broth at each of a series of temperatures, plating with and without acriflavine as before, and incubating the plates at 35°C. Rate constants calculated from the results of nine such experiments are presented in the form of an Arrhenius plot in Fig. 4. The temperature effect corresponds to a $\Delta H\ddagger$ of about 16 kcal/mole between 27 and 39°C, and to the higher value of about 60 kcal/mole below 25°. Although the significance of this

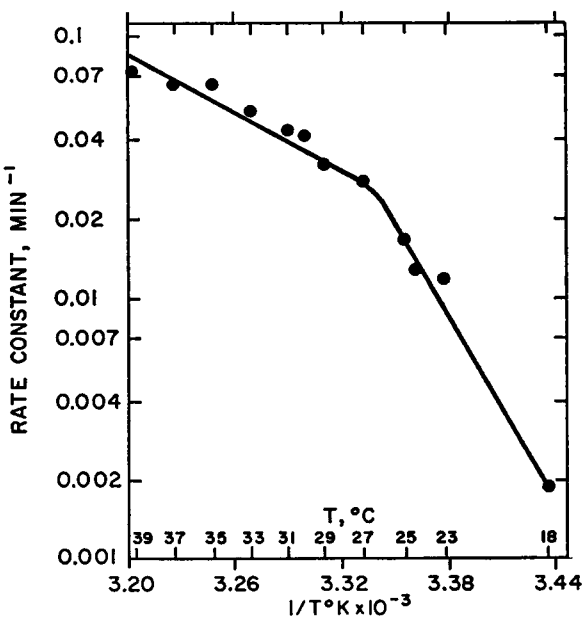

FIGURE 4 Arrhenius plot for the recovery of irradiated Rd Str Nb cells from acriflavine sensitization.

change is not understood here, similar effects are known in other systems for both single enzyme reactions and physiological processes involving many such reactions (17).

Intracellular Reactivation of Genetic Markers

It was expected, on the basis of the foregoing, that irradiated cells should be able to repair some of the damage done to genetic markers contained within them if the recovery process were to involve repair of DNA. To test this possibility Rd Str Nb cells were grown without aeration in BHHN broth at 35°C to a concentration of 4×10^8/ml and frozen in 15% glycerine. For the experiments, the cells were thawed, washed twice in TCNT, concentrated by a factor of two, and irradiated with 325

ergs/mm² UV. The cells were then centrifuged and resuspended in 35°C BHHN broth. After various times of incubation, cells were removed and lysed, and their DNA was assayed for transforming activity as described in Methods and Materials. The results are shown in Fig. 5. Four other experiments gave similar results.

As is seen, both the Str and Nb markers can be reactivated intracellularly in donor bacteria, but to observe this reactivation for the Nb marker, acriflavine must be used to stop reactivation in the Rd recipient cells. In the case of the Str marker,

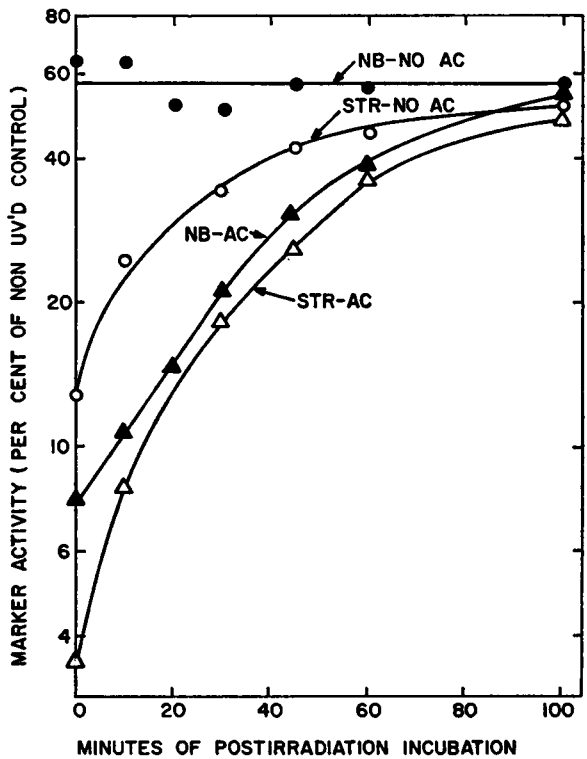

FIGURE 5 Per cent marker activity (Str and Nb) as a function of time of postirradiation incubation in broth. Lysates made from samples which were taken at various times of incubation were used to transform Rd cells both in the presence (AC) and absence of acriflavine (no AC).

where recipient cells are unable to repair the damage as effectively (4), reactivation in donor bacteria can be observed even in the absence of acriflavine. The figure shows that at the dose given and under the conditions used, the irradiated cells can reactivate approximately 45% of the activity of both markers (corresponding to a dose modification factor of 0.1–0.15 in four experiments), but that the recipient repairs only about 10% of the Str activity, while it repairs 50% of the Nb activity.

The shape of these curves can be described by an equation derived in much the

same manner as equations 1 and 2, but taking into account the peculiar inactivation curve shapes for transforming DNA. This curve shape is adequately accounted for by the target size population expected from random recombinations between the donor DNA with the recipient cell's genome, as discussed by several authors (18, 19, 20), but something less than the complete model is required for our purposes. We need only assume here (in agreement with the model) that the mean number of inactivating hits per unit length, h, is initially proportional to the UV dose. This, with the empirical inactivation law for transforming DNA (21) makes the survival

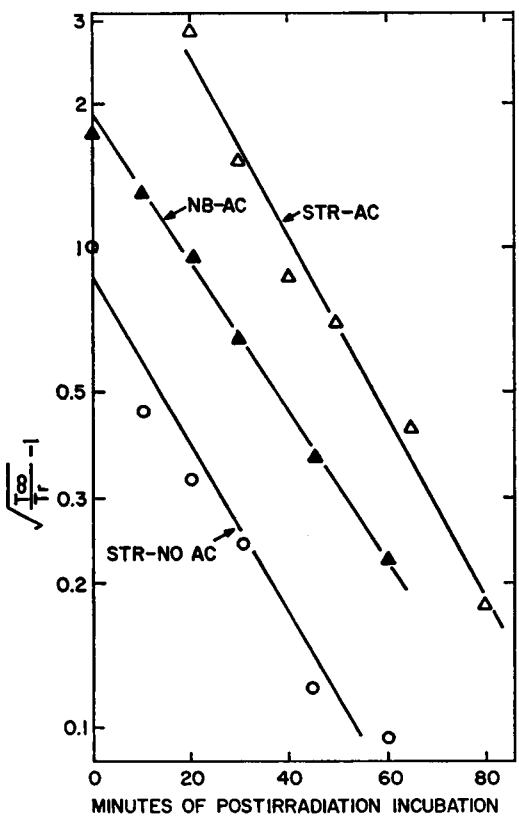

FIGURE 6 Log $[\sqrt{(T_\infty/T_r)} - 1]$ as a function of time of postirradiation incubation for the data of Fig. 5. The data for the Str marker assayed with acriflavine is shifted 20 min to the right for clarity. T_∞ is the number of transformants at infinite time of reactivation and T_r is that at any time of reactivation.

of marker activity $T/T_0 = 1/(1 + ch)^2$, and if we assume this relation remains true of DNA in which hits have been randomly erased by repair, we obtain $T_r/T_\infty = 1/(1 + ch\, e^{-kt})^2$, where T_r is the number of transformants after a given UV dose and after a time t of reactivation with rate constant k. T_∞ is the number of transformants at infinite reactivation time, and c is a constant. Then

$$\log [(T_\infty/T_r)^{1/2} - 1] = \log ch - kt. \tag{3}$$

Plotting the left side of equation 3 vs. time gives a straight line whose slope is a

measure of the rate constant. Fig. 6 shows the data of Fig. 5 plotted in this way. The average rate constant is 0.037 ± 0.005 per minute, which is approximately half of that for the reactivation of colony-forming ability at the same temperature. The fact that two such different UV doses (19 and 325 ergs/mm^2) result in roughly similar values for the rate constant encourages us to believe that k has something of the meaning we intend.

The fact that the Nb marker activity does not increase when no acriflavine is used indicates that no appreciable residual DNA synthesis occurred during the incubation period. In two other experiments we have found that the total DNA (indole reactive material) does not increase either. Thus it appears that reactivation is being observed.

DISCUSSION

The sensitizing effect of acriflavine on irradiated *H. influenzae* is thought to be due to the inhibition of a dark repair process since postirradiation incubation of the cells resulted in decreased susceptibility of the colony-forming ability to depression by acriflavine.

One can calculate a first-order rate constant of 0.03 per minute for the excision of thymine dimers from *E. coli* in a minimal medium at 37°C from the data of Setlow and Carrier (12). Similarly when equation 2 is used to fit two curves obtained by Harm (22) on liquid holding recovery in *E. coli* B and B/r at 22°C, a rate constant of 0.016 per minute can be calculated. In the present work, rate constants of 0.065 and 0.012 per minute were obtained at 37°C and 23°C, respectively.

An interesting facet of the results of the temperature-dependence experiments is the comparison with the temperature work of Voll and Goodgal (23) on recombination in *H. influenzae*. They found that recombination occurred between donor Str and recipient Nb25 markers, between 27 and 40°C, but that at 17 and 20°C, recombination was experimentally unobservable. Both of these temperatures are below the break seen in the Arrhenius plot in Fig. 4. It is possible that the enzyme(s) responsible for recovery of colony-forming ability of irradiated cells from acriflavine sensitivity could be operative in recombination. This is not a new idea. The rec$^-$ strains of *E coli*, which are unable to act as recipients in conjugation are also UV-sensitive (24).

The finding that *H. influenzae* is able to repair two genetic markers which are irradiated intracellularly as part of the chromosome of the cell to approximately the same extent is in contrast to the results of Patrick and Rupert on extracellularly irradiated DNA (4). Their results clearly show that when the DNA bearing the markers is irradiated extracellularly and then used to transform cells the Nb marker is far more resistant to UV than is the Str marker. The results, obtained by using acriflavine during the transformation assay, suggest that the majority of the difference is due to a preferential repair of the Nb marker by the recipient cell. We have confirmed this result. There are at least two possibilities to account for this differ-

ence. The first is that repair occurs after pairing of the donor DNA with that of the recipient. Notani and Goodgal (25) found that only one strand of the transforming marker duplex is integrated into the recipient genome. Voll and Goodgal (23) showed that, upon uptake, the biological activity of the Str marker suffers a substantially greater decrease than that of the Nb marker during the transformation reaction and suggested that the Str marker is a more complex (i.e. multisite) mutation than the Nb which probably arose from a single-step mutation. If an excision-resynthesis type of repair were to occur after pairing of the donor DNA with that of the recipient, the UV-damaged portion of the donor DNA would be excised, and the resynthesis template would necessarily be recipient DNA. Thus, if the marker were excised, the reconstructed region would bear no marker. If the Str marker had a larger or multisite "target size" for the excision process, it would appear to be more UV-sensitive than the Nb marker. In the case in which cells bearing the markers are irradiated and then incubated to allow repair, the repair template would bear the marker in complementary form, and no "repairing out" of the markers could occur. Here the question of marker complexity would not affect the repair, and both the markers would be repaired to similar extents.

The second hypothesis is that UV enhances the mispairing of the mutant marker with the homologous region of the recipient cell more in the case of the Str marker than in that of the Nb marker, and that there is an enzyme which recognizes the mispairing and preferentially destroys the Str marker thus rendering it unsusceptible to repair. The destructive agent might be the enzyme(s) responsible for the difference in the integration efficiencies of the two markers.

The first hypothesis is questionable because it predicts that excision would occur over a very long distance. At a biologically effective dose to transforming DNA of 1000 ergs/mm^2, for example, there is about one dimer per 1000 base pairs of *Hemophilus influenzae* DNA (26). If a marker (considered here as a point mutation within a gene) were to be excised out, excision would be required to occur for a distance of 500 nucleotides (on the average) on a single strand. In *E. coli*, Setlow and Carrier (12) have estimated the length of the excised region to be 30 nucleotides. The length of this region in *Hemophilus* is not known.

During this investigation, it was observed that competent *H. influenzae* cells were not able to repair the Str and Nb markers (when contained as part of the genome of the cell) as well as log phase cells grown without aeration. Kelner (27) discovered that irradiated competent *Bacillus subtilis* cannot be photoreactivated whereas noncompetent cells can. These findings suggest that repair enzymes may not be as numerous or as active in competent cells, or that the DNA of the cell is relatively inaccessible to the repair enzymes.

Experiments similar to our intracellular reactivation of transforming DNA experiments have been done by Stuy (28). However, he did not use acriflavine and had the difficulty of extensive cell lysis during incubation of irradiated cultures of

H. influenzae. Our irradiated cultures showed little (25–30%) lysis as determined by Coulter counter (Coulter Electronics, Chicago, Ill.) in 3½ hr of incubation. Moreover, in all of Stuy's experiments considerable growth of cells was observed upon incubation of irradiated cells. Our cultures stopped growing immediately. These complications make difficult a comparison of his data with our own.

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REFERENCES

1. WITKIN, E. 1963. *Proc. Natl. Acad. Sci. U.S.* **50**:425.
2. ALPER, T. 1963. *Nature.* **200**:534.
3. DOUDNEY, C., B. WHITE, and B. BRUCE. 1964. *Biochem. Biophys. Res. Commun.* **15**:70.
4. PATRICK, M., and C. RUPERT. 1967. *Photochem. Photobiol.* **6**:1.
5. HARM, W. 1967. *Mutation Res.* **4**:93.
6. FEINER, R., and R. HILL. 1963. *Nature.* **200**:291.
7. OKUBO, S., and W. ROMIG. 1965. *J. Mol. Biol.* **14**:130.
8. REITER, H., and B. STRAUSS. 1965. *J. Mol. Biol.* **14**:179.
9. MAHLER, I. 1965. *Biochem. Biophys. Res. Commun.* **21**:384.
10. SETLOW, R. 1964. *J. Cellular Physiol.* **64** (No. 2, Suppl. 1):51.
11. SETLOW, R., W. CARRIER, and R. WILLIAMS. 1967. *Biophys. Soc. Abstr.* 83.
12. SETLOW, R., and W. CARRIER. 1964. *Proc. Natl. Acad. Sci. U.S.* **51**:226.
13. BOYCE, R., and P. HOWARD-FLANDERS. 1964. *Proc. Natl. Acad. Sci. U.S.* **51**:293.
14. BOLING, M., S. MODAK, G. PRICE, A. MATTINGLY, M. GORDON, and J. SETLOW. 1968. *Biophys. J. Soc. Abstr.* **8**:A-54.
15. BARNHART, B., and R. HERRIOTT. 1963. *Biochim. Biophys. Acta.* **76**:25.
16. ROBERTS, R., and E. ALDOUS. 1949. *J. Bacteriol.* **57**:363.
17. JOHNSON, F., H. EYRING, and M. POLISSAR. 1954. The Kinetic Basis of Molecular Biology. John Wiley & Sons, Inc., New York. pp. 200-214.
18. RUPERT, C. 1961. *J. Cellular Physiol.* **58**(Suppl. 1):89. In discussion of paper by A. H. Doermann, p. 79.
19. BRESLER, S., V. KALININ, and D. PERUMOV. 1967. *Mutation Res.* **4**:389.
20. RUPERT, C. 1968. *Photochem. Photobiol.* **7**:437.
21. RUPERT, C., and S. GOODGAL. 1960. *Nature.* **185**:556.
22. HARM, W. 1966. *Photochem. Photobiol.* **5**:747.
23. VOLL, M., and S. GOODGAL. 1965. *J. Bacteriol.* **90**:873.
24. HOWARD-FLANDERS, P., and L. THERIOT. 1966. *Genetics.* **53**:1137.
25. NOTANI, N., and S. GOODGAL. 1966. *J. Gen. Physiol.* **49** (6, Pt. 2):197.
26. SETLOW, R., and W. CARRIER. 1966. *J. Mol. Biol.* **17**:237.
27. KELNER, A. 1965. *Radiation Res.* **25**:205.
28. STUY, J. H. 1959. *J. Bacteriol.* **78**:49.