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Mechanism of Caffeine Enhancement of Mutations Induced by Sublethal Ultraviolet Dosages

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Certain chemical compounds increase mutation frequency of *Escherichia coli* B/r significantly when used in conjunction with nonlethal ultraviolet (UV) dosages. Studies were done to elucidate the mechanism of this enhancing mutational effect. Dark survival curves showed that 500 μg of caffeine per ml in the postirradiation medium markedly decreased survival to 60 ergs/mm² of UV in strain B/r. Caffeine did not markedly decrease survival to UV in strain B/r WP-2 *hcr*⁻. At least 90% of the mutations induced to streptomycin resistance by UV and 85% of those induced by UV with caffeine could be photoreversed. Experiments with thymine analogues suggested that thymine dimerization at the streptomycin locus was the primary premutational photoproduct induced by sublethal UV dosages. Caffeine did not interfere with the photoreversal of induced mutants, indicating that it probably does not bind to the photoreactivating enzyme or to a UV-induced lesion in the DNA. Addition of DNA or irradiated DNA with 500 μg of caffeine per ml resulted in no loss of the caffeine activity. The excision of UV-induced thymine-containing dimers from *E. coli* B/r T⁻ was investigated in the presence and absence of caffeine. Our results indicated that caffeine prevents excision of thymine dimers, presumably by binding to the excising enzyme. This binding results in an impairment of repair, which produces the increase in mutant numbers.

Caffeine, theophylline, and theobromine, as well as acridine dyes, increase the frequency of mutations induced by lethal and sublethal ultraviolet (UV) light when they are added to the postirradiation medium of *Escherichia coli* strain B/r (3, 14, 23, 29, 30). Bacterial killing is increased when bacteria irradiated with lethal and sublethal dosages are plated on a medium containing acriflavine (1, 7, 30) or caffeine (7, 17, 29). These compounds decrease the survival of UV-irradiated phage by inhibiting host cell reactivation (5). The increase in both killing and mutation can be attributed to inhibition of a "dark repair" process that repairs UV photoproducts in the irradiated bacteria. Irradiation of bacteria with high dosages of UV light produces a variety of pyrimidine dimers in the deoxyribonucleic acid of these organisms (DNA) (21). Pyrimidine dimers can be repaired either by photoreactivation, in which the photoreactivating enzyme splits dimers in situ (35), or by excision of pyrimidine dimers from the DNA in the dark (2, 20, 25). Numerous UV-sensitive mutants of *E. coli* have been isolated and found to be defective in their ability to excise dimers upon incubation in the dark (2, 9, 20). These mutants were also found to be incapable of repairing UV-irradiated T1 phage (7, 18). Genes controlling "dark repair" have been mapped in bacteria (6, 8, 9).

Caffeine, theophylline, and theobromine increase the mutation frequency to high levels of streptomycin resistance significantly when used in conjunction with sublethal UV dosages (3, 23). The mechanism of action of these compounds in the mutational enhancement is not completely understood. This study explores the roles played by photoproducts of UV light in the mechanism of "mutational synergism" or "mutation enhancement" between sublethal dosages of UV light and caffeine.

Our operational definition of "nonlethal" or "sublethal" UV dosage is that range of dosages which results in 100% survival when cells are subsequently plated on appropriate growth media. It is recognized, of course, that there may still be some undetected deaths in the irradiated population, but these, if they occur, are not detectable by usual microbiological procedures.

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MATERIALS AND METHODS

Bacterial strains. Radiation-resistant *E. coli* strain B/r, obtained from E. Witkin, was employed in some of the experiments. *E. coli* strain B/r T⁻, a thymineless mutant of B/r, was isolated in our laboratory. *E. coli* strain B/r WP-2 *hcr*⁻, a radiation-sensitive mutant of *E. coli* B, originally isolated by Ruth Hill, was obtained from C. H. Clarke. Stock cultures of these organisms were transferred biweekly on slants of chemically defined medium (M-9) supplemented with required growth factors as necessary.

Induction and scoring of mutants. For the induction and scoring of streptomycin-resistant mutants, the procedure of Shankel (23) was used. Replicate populations of cells were prepared by impinging cells on membrane filters obtained from Carl Schleicher & Schuell Co., Keene, N.H. The membranes were placed on cold phosphate-buffered agar plates, exposed to 60 ergs/mm² of UV irradiation (unless otherwise noted), transferred to prewarmed (37 C) plates of soft (0.8% agar) Difco Brain Heart Infusion (BHI) with or without 500 μg/ml of caffeine, and incubated at 37 C until maximal phenotypic expression occurred, usually 3.5 hr. The membranes were then transferred to soft BHI agar plates containing 1 mg of Squibb dihydrostreptomycin sulfate per ml for selection of the mutants. After an additional 48 hr of incubation (37 C), the number of high-level streptomycin-resistant mutants was determined by scoring the number of colonies which developed in the presence of the antibiotic.

Photoreactivation. Cells impinged on membrane filters were placed on phosphate-buffered agar plates, with or without caffeine, and subjected to UV irradiation. The plates were then placed immediately on an ice bath and exposed to light from two 300-w photoflood lamps at a distance of 37 cm for a period of 40 min. Pairs of membranes were then transferred to BHI agar or BHI-caffeine agar plates to allow mutation fixation and subsequent phenotypic expression. Selection was on BHI-streptomycin plates.

Specific methods for labeling and excision experiments. The DNA of *E. coli* B/r T⁻ was labeled with ³H-thymine by growth in M-9 medium containing 1 mg of Vitamin Free Casamino Acids per ml and 2 μg of methyl-³H-thymidine per ml (New England Nuclear Corp., Boston, Mass.). It had a specific activity of 17.1 c/mM. The cells were incubated at 37 C in a water-bath shaker to late log phase, washed three times in M-9, and suspended in M-9 medium for irradiation. The UV treatments were performed with a 15-w General Electric germicidal lamp which predominantly emitted a wavelength of 2,537 A. All operations following irradiation were carried out under yellow light to minimize photoreactivation. Samples (30 ml) of M-9 containing 6×10^7 cells/ml were irradiated in 14-cm petri dishes with shaking. The dose rate was 20 ergs/mm² per sec as determined by a General Electric germicidal UV meter. The dosage, unless otherwise noted, was 600 ergs/mm². Biological dosimetry indicated that this dosage was essentially the same as 60 ergs/mm² to cells on the surface of membrane filters. Five 30-ml samples were treated as follows: (i) no UV followed by 2-hr incubation at 37 C in M-9 supplemented with thymine (2 μg/ml) and Vitamin Free Casamino Acids (1 mg/ml); (ii) UV and no incubation, i.e., chilled immediately in an ice-bath; (iii) UV followed by 2-hr incubation in M-9 supplemented with thymine and Casamino Acids; (iv) UV followed by 2-hr incubation in M-9 supplemented with thymine, Casamino Acids, *and* caffeine (1,000 μg/ml); (v) UV followed by 2-hr incubation in M-9 containing thymine, Casamino Acids, and acriflavine (5 μg/ml). All postirradiation treatments were carried out in the dark to prevent photoreactivation. The samples were then centrifuged, washed, suspended in 1 ml of cool 5% trichloroacetic acid, and held in ice water for 50 min. The samples were then centrifuged and the acid-soluble fractions decanted. The acid-insoluble fraction was washed in 0.5 ml of cold trichloroacetic acid and centrifuged. The supernatant fluids were combined and dried under a stream of warm air. The samples were hydrolyzed with concentrated trifluoroacetic acid at 170 C for 90 min, and were paper chromatographed in *n*-butyl alcohol-acetic acid-water (200:30:75), according to the procedure of Boyce and Howard-Flanders (2). The strips were dried at room temperature and scanned for ³H activity in a 4π gas-flow chromatogram scanner.

Incorporation of thymine analogues. E. coli B/r T⁻ was grown in M-9 medium containing 0.2% sulfanilamide (26), supplemented with 200 μg of 5-bromouracil or iodouracil per ml, or 20 μg of azathymine per ml, and was incubated for 24 hr with shaking at 37 C. The total number of mutants induced and expressed with UV and caffeine was tested by preparing replicate populations of 5×10^8 cells on membrane filters. In all experiments, unless otherwise noted, the cells were exposed to 60 ergs/mm². For the irradiation, phenotypic expression, selection, and scoring of induced streptomycin-resistant mutants, we used the procedure of Shankel (23).

All results reported in this paper represent an average of at least three independent experiments, each of which followed the same pattern.

RESULTS

Wulff (34) previously showed that low UV dosages of the type employed here do produce thymine dimers. The finding that many strains of of *E. coli*, differing in their sensitivity to UV, all have equivalent numbers of thymine dimers produced in their DNA by the same UV dosages (22) supports the assumption that the same amount of damage is produced in the two strains B/r and WP-2 *hcr*⁻ and that the major difference between these strains is their ability to excise the UV photoproducts. Thus, the sensitivity of strain *hcr*⁻ appears to be due to reduced repair of UV damage, resulting from the failure of normal excision (9). Experiments were done to determine whether caffeine would enhance lethality and

mutagenesis in WP-2 *hcr*$^-$ and in B/r, which retains excision ability. The survival values (Table 1) show that different types of responses were obtained in the two types of strains when plated on media containing caffeine. Caffeine decreased survival of strain B/r to normally nonlethal and lethal UV dosages when incorporated in the postirradiation medium. Caffeine only slightly altered survival to UV in strain WP-2 *hcr*$^-$. This suggested that caffeine increases lethality by interfering with photoproducts that are excisable in B/r. Furthermore, the absence of mutation enhancement (Table 1) in the strain lacking excision ability indicated that caffeine interferes with excision of photoproducts in strain B/r, and this interference produces the increase in mutant numbers. If nonexcisable photoproducts were involved in the synergistic (enhancement) effect, differences in the excision ability would be relatively unimportant, and the frequency of induced mutations to streptomycin resistance would be expected to be similar on the two media.

We next investigated whether the mutations to streptomycin resistance, induced by UV with caffeine, are primarily due to the thymine dimer or to another photoproduct. It has been reported that certain mutations to prototrophy are photoreversible in an *E. coli phr*$^-$ strain. Conversely, photoreversal of UV-induced mutations to streptomycin resistance, as well as UV killing, fails to occur in this strain (11, 31, 33). We found that photoreactivation of *E. coli* B/r (Fig. 1) results in repair of over 90% of the premutational damage induced by nonlethal dosages of UV and repair of 85% of the damage induced by combinations of UV and caffeine. This finding indicates that most of the mutations induced by combinations of UV and caffeine are photoreversible, and is in agreement with a report by Shankel (23).

However, photoreversibility of mutations by the photoreactivating enzyme does not provide any information concerning the specific type of pyrimidine dimers involved. Studies by Setlow (21), employing a homopolymer complex of polydeoxyinosinic acid:polydeoxycytidylic acid, indicate that cytosine dimers are created upon UV irradiation at lethal dosages and are destroyed by visible light in the presence of photoreactivating enzyme. To ascertain whether thymine dimers are involved in these mutations, experiments with thymine analogues were initiated. Mutational synergism (Table 2) was significantly reduced in cells in which 5-bromouracil, 5-iodouracil, or 6-azathymine was substituted for thymine. This indicates the importance of thymine in the induction of streptomycin-resistant mutations by sublethal UV dosages with caffeine. The experiments with 6-azathymine suggested that thymine dimerization is the main photoproduct induced at sublethal UV dosages leading to streptomycin resistance, since 6-azathymine does not dimerize (28). Dark survival curves showed that caffeine appreciably decreases survival following UV irradiation in normal B/r cells. At 60 ergs/mm^2, there was approximately 15 to 20% killing in the presence of caffeine. Caffeine enhanced lethality to a lesser extent in 6-azathymine substituted cells. At the same UV dosages, there was only 5% killing (Fig. 2). These experiments suggested that thymine dimerization is the main prelethal photoproduct induced at normally *sublethal* UV dosages.

Acriflavine and acridine orange increase the lag in DNA synthesis and decrease the rate of thymine dimer excision (19). Acriflavine (1 μg/ml) strongly inhibits DNA, ribonucleic acid, and protein synthesis (4). We studied the effect of caffeine and acriflavine on the rate of thymine dimer excision in *E. coli* B/r T$^-$ (Fig. 3). The results indicate that caffeine prevents excision of the thymine dimer in the dark in a B/r T$^-$ strain. Acriflavine was found to produce a similar effect.

TABLE 1. *Relative population survival and phenotypic expression of UV-induced mutants on BHI and BHI-caffeine (BHI-caf) in strains WP-2 hcr$^-$ and B/r at UV doses resulting in high survival*[a]

Strain	UV dosage (ergs/mm^2)	Percentage survival		No. of mutants	
		BHI	BHI-caf	BHI	BHI-caf
WP-2 hcr$^-$	0	100	100	4	3
	3	95	94	211	171
	6	90	84	195	83
	9	29	23	77	54
B/r	0	100	100	2.5	2.5
	24	100	100	74	420
	48	100	85	65	205
	72	94	76	83	137

[a] The starting population in each case was 3×10^8 cells. These experiments were carried out in the M.R.C. Mutagenesis Research Unit, Edinburgh, Scotland. Cells were grown in M-9 supplemented with 0.01% glucose and 50 μg of tryptophan per ml. Conditions were as described in Materials and Methods, with the following exceptions: irradiation was with a Phillips 15-w TUV tube giving 90% of its output at 2537 A; UV measurements were made with a "Jagger" meter; Oxoid BHI, agar, and membranes were utilized; caffeine was obtained from British Drug Houses; and Glaxo dihydrostreptomycin sulphate was the selective agent.

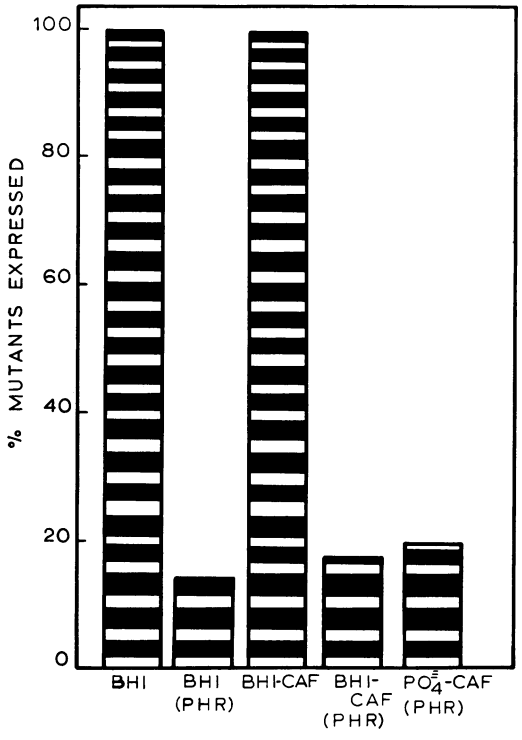

FIG. 1. *Influence of postirradiation medium on the efficiency of photoreversal of induced potential mutations in E. coli B/r when exposure to photoreactivating light follows immediately after UV irradiation. The total numbers of mutants produced in controls on BHI agar and on BHI-caffeine agar are indicated as 100%. BHI, medium after irradiation was BHI agar; BHI-PHR, medium during photoreactivation was phosphate-buffered agar, medium after photoreactivation was BHI agar; BHI-CAF, medium after irradiation was BHI-caffeine agar; BHI-CAF-PHR, medium during photoreactivation was phosphate-buffered agar, medium after photoreactivation was BHI-caffeine agar; PO_4-CAF-PHR, medium during photoreactivation was phosphate-buffered agar containing caffeine, medium after photoreactivation was BHI-caffeine agar. Starting populations were 5 × 10^8 cells on each membrane filter.*

These results agree with our previous observations and with the theory that caffeine increases the sublethal UV-induced frequency of mutations to streptomycin resistance mainly by preventing excision of the thymine dimers.

The mode of action of the caffeine molecule in blocking excision is not known. In the search to find additional compounds exhibiting the synergistic effect, we investigated a number of dyes which are known to combine with DNA in vitro. There is some evidence that acridines and caffeine attach to DNA in vitro (12, 13, 27). Using an auxotrophic to prototrophic mutation system, Witkin (30) showed that acriflavine and other dyes which are known to combine with DNA in vitro show a similar effect to that of caffeine when used in conjunction with low UV dosages. Our results showed that acriflavine also increases the number of mutants to high-level streptomycin resistance appreciably when used in conjunction with UV dosages (Table 3). The other dyes did this to a lesser extent. If DNA is added to the medium containing an appropriate concentration of acriflavine (Table 4), the number of streptomycin-resistant mutants is decreased, possibly due to binding of the dye to the DNA. Nevertheless, addition of DNA or irradiated DNA (2 mg/ml) with 500 μg of caffeine per ml resulted in no loss of the caffeine activity. Lower concentrations of DNA produced a graded effect with acriflavine, but were also ineffective with caffeine.

TABLE 2. *Effects of thymine analogues on the induction of mutations to UV light and caffeine in E. coli B/r T⁻*[a]

Analogue	Postirradiation expression medium	No. of mutants[b] expressed per 5 × 10^8 cells
5-Bromouracil	BHI	18
	BHI-caf	19
5-Iodouracil	BHI	20
	BHI-caf	22
6-Azathymine	BHI	15
	BHI-caf	17
Thymine	BHI	55
	BHI-caf	375

[a] *E. coli* B/r T⁻ was grown in M-9 medium supplemented with 2 mg of sulfanilamide per ml and 200 μg of bromouracil or iodouracil per ml or 20 μg of azathymine per ml, and was incubated for 24 hr with shaking at 37 C.

[b] The total number of induced mutants expressed was tested by preparing replicate populations of 5 × 10^8 cells on membrane filters. The membranes bearing the cells were irradiated with 60 ergs/mm² and then were placed on either BHI or BHI-caffeine (BHI-caf) agar plates to complete expression before transfer to BHI-streptomycin for selection of the mutants.

DISCUSSION

Significant numbers of mutations can be induced in *E. coli* B/r by doses of UV light so small that there is no measurable decrease in the number of viable cells after irradiation (16). The dose range for the nonlethal but mutagenic UV was found to extend from 10 to 60 ergs/mm², for high-level streptomycin resistance in *E. coli* B/r (24). After UV irradiation, the number of

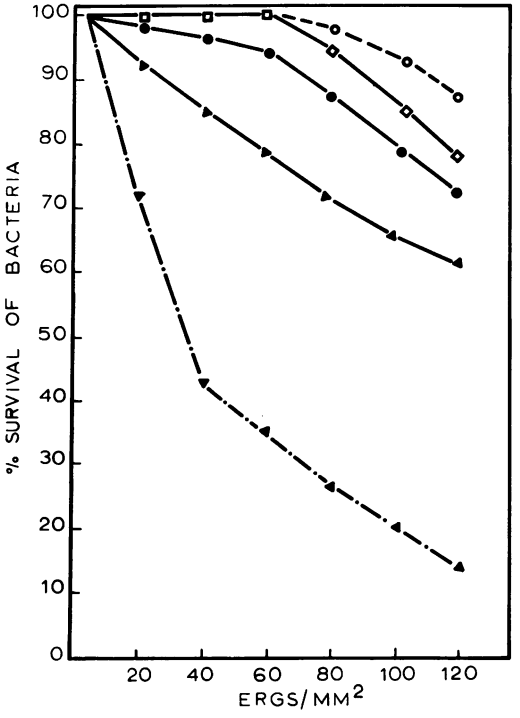

FIG. 2. *Effect of thymine analogues on the survival of E. coli B/r T⁻ to UV light and caffeine. Symbols: □, irradiated and plated on BHI agar; △, irradiated and plated on BHI-caffeine agar; ○, grown in the presence of 6-azathymine, irradiated and plated on BHI agar; ●, grown in the presence of 6-azathymine, irradiated and plated on BHI-caffeine agar; ▲, grown in the presence of 5-bromouracil, irradiated and plated on BHI agar.*

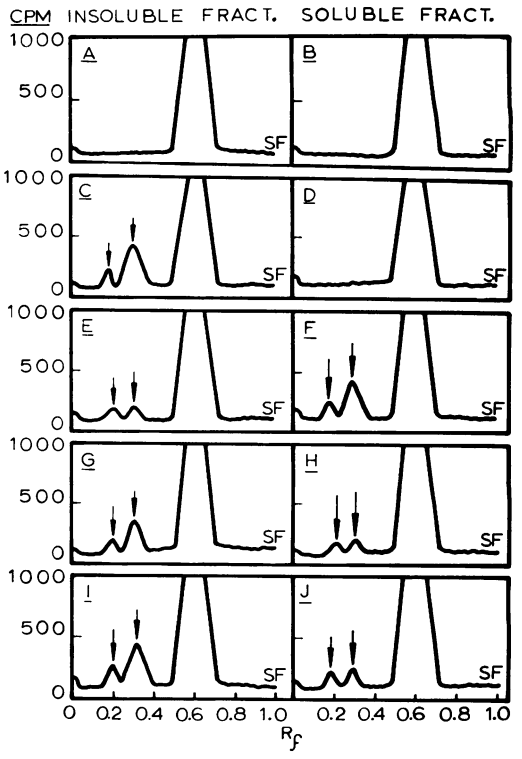

FIG. 3. *Radiochromatograms showing distribution of radioactivity in the acid-insoluble and -soluble fractions of irradiated and unirradiated E. coli B/r T⁻. Cells were labeled with ³H-thymine. The UV dosage applied was 600 ergs/mm². Treatments for cells shown in individual chromatograms were as follows: A and B were unirradiated controls incubated in M-9 plus thymine and Casamino Acids for 2 hr in the dark; C and D were irradiated, but not incubated in the dark; E and F were irradiated and incubated in M-9 plus thymine and Casamino Acids for 2 hr in the dark; G and H were irradiated and incubated in M-9 plus Casamino Acids, thymine, and caffeine (1,000 μg/ml) for 2 hr in the dark; I and J were irradiated and incubated in M-9 plus thymine, Casamino Acids, and acriflavine (5 μg/ml) for 2 hr in the dark. The major peak has the same R_F value as unirradiated thymine; the minor peak has the same R_F as the thymine dimer.*

streptomycin-resistant mutants can be greatly increased by plating in a medium containing caffeine or acriflavine.

A comparison of lethality and mutagenesis in WP-2 *hcr⁻*, a UV-sensitive strain which lacks excision ability, and in strain B/r, which retains excision ability, shows that potentially excisable photoproducts participate in the induction of mutations to streptomycin resistance. When caffeine is incorporated in the postirradiation medium, it decreases survival and increases the frequency of mutation to streptomycin resistance resulting from otherwise nonlethal UV dosages in B/r. However, it has little effect on mutation frequency or survival in strain WP-2 *hcr⁻*. Our data are compatible with the idea that the major difference between these two strains is their ability to excise UV photoproducts (22) and that caffeine interferes with the dark repair mechanism (14, 15).

At least 90% of the mutations induced to streptomycin resistance by UV light and 85% induced by UV light with caffeine can be photoreversed, suggesting that most of the mutations to streptomycin resistance observed in the resistant strain are due to pyrimidine dimers (32). The fact that most mutations induced by combinations of UV light and caffeine are photoreversible suggests that these mutations are produced by a mechanism similar to that which induces them in the absence of caffeine.

Photoreactivation alone is not an adequate

TABLE 3. *Effects of dyes on the development of mutations induced by nonlethal dosages of UV lights*

Compound	No. of mutants[a] expressed per 5×10^8 cells
Acriflavine[b]	558
Methylene blue[c]	210
Acridine orange[d]	288
Malachite green[e]	185
Toluidine blue[f]	218
Control (UV only)	70

[a] The total number of induced mutants was tested by preparing replicate populations of 5×10^8 cells of *E. coli* B/r on membrane filters. The membranes bearing the cells were irradiated with 60 ergs/mm² and then were placed on either BHI or BHI-dye agar plates to complete expression before transfer to BHI-streptomycin for selection of the mutants.

[b] Acriflavine, 3 μg/ml.

[c] Methylene blue, 4 μg/ml.

[d] Acridine orange, 5 μg/ml.

[e] Malachite green, 5 μg/ml.

[f] Toluidine blue, 2.5 μg/ml.

TABLE 4. *Effects of UV irradiated and nonirradiated sodium deoxyribonucleate (Na-DNA 2 mg/ml) on the development of mutations induced by nonlethal dosages of UV light and caffeine or acriflavine*

Compound	No. of mutants[a] expressed per 5×10^8 cells	Irradiated Na-DNA added	Nonirradiated Na-DNA added
Acriflavine[b]	510	235	230
Caffeine[c]	380	388	385
Control (UV only)	58	70	72

[a] The total number of induced mutants expressed was tested by preparing replicate populations of 5×10^8 cells of *E. coli* B/r on membrane filters. The membranes bearing the cells were irradiated with a dose of 60 ergs/mm² and then were placed on either BHI, BHI-caffeine, or BHI-acriflavine, with or without sodium deoxyribonucleate, to complete expression before transfer to BHI-streptomycin for selection of the mutants.

[b] Acriflavine, 0.5 μg/ml.

[c] Caffeine, 500 μg/ml.

criterion for determining the type of pyrimidine dimers (10). Further evidence on the nature of the lesion leading to streptomycin resistance was derived from our experiments with thymine analogues. Experiments with thymine analogues indicate that thymine dimerization at the streptomycin locus is the primary premutational photoproduct induced by sublethal UV dosages, but not the sole prelethal photoproduct. Apparently another lesion besides thymine dimer is induced at sublethal UV dosages. Caffeine also interferes with the repair of this lesion. This is not suprising since survival is probably influenced by the production and repair of potentially lethal UV damage anywhere in the DNA, whereas the frequency of a given type of mutation is more likely to be affected by the production of UV photoproducts in a particular base pair or a small group of base pairs.

In a thymine-requiring mutant of B/r, caffeine has been shown to prevent excision of thymine dimers in the dark. A similar effect has been demonstrated for acriflavine (19).

Since the photoreactivating enzyme can photoreverse UV-induced lesions in the presence of caffeine and since addition of DNA does not reverse the caffeine effect, it seems that the caffeine molecule does not bind to the UV lesion in the DNA. It is more probable that caffeine enhances the frequency of mutations by competing successfully with the thymine dimers for excision enzyme, thus resulting in an impairment of repair, which in turn results in the increase in mutant numbers.

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