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MUTATIONAL SYNERGISM BETWEEN RADIATIONS AND METHYLATED PURINES IN *ESCHERICHIA COLI*

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ABSTRACT

DONESON, IRA N. (University of Kansas, Lawrence), AND DELBERT M. SHANKEL. Mutational synergism between radiations and methylalted purines in *Escherichia coli*. J. Bacteriol. **87**: 61-67. 1964.—A synergistic mutational effect was demonstrated between low doses of ultraviolet light and the methylated purines caffeine, theophylline, and theobromine. Caffeine produced the greatest effect and theobromine the least effect. The magnitude of the synergism was inversely related to the ultraviolet dosage. A large percentage of the synergistic effect could be "photoprevented" by exposure of the ultraviolet-treated cells to white light prior to exposure to the analogues. The consequence of the combined treatment occurred only when the chemical treatment followed the ultraviolet treatment. Furthermore, it was necessary to administer the chemical treatment soon after the ultraviolet treatment or the mutants were "lost." When cells were treated with low dosages of ultraviolet light and of X irradiation (X ray), the result was merely additive, and combinations of X ray and chemical treatment yielded no synergism. Synchronous growth studies indicated that a particular growth stage of the organisms was most susceptible to the synergistic effect. The mutation studied was that of *Escherichia coli* B/r to high-level streptomycin resistance.

Caffeine, theophylline, and theobromine constitute a group of chemically related methylated purines. Novick and Szilard (1951) showed that caffeine is mutagenic for bacterial cells. More recently, Gyorffy (1960) demonstrated that caffeine treatment increases the number of streptomycin-resistant mutants occurring in cultures of *Xanthomonas phaseoli*. Witkin (1959) noted an increase in the number of reversions from auxotrophy to prototrophy when ultraviolet-induced mutants were allowed to develop in a caffeine-containing medium. This phenomenon was explored extensively by Lieb (1961), who demonstrated that theophylline produced a similar effect and determined many characteristics of the phenomenon.

The development of mutations when *Escherichia coli* B/r is subjected to nonlethal amounts of ultraviolet light was reported by Matney, Shankel, and Wyss (1958). It was subsequently found (Shankel, 1961) that a large increase in mutant numbers was obtained when mutations to high-level streptomycin resistance, produced by nonlethal ultraviolet dosages, were phenotypically expressed in the presence of 500 μg/ml of caffeine. Further study (Shankel, 1962) demonstrated that the synergistic effect was (i) not reversed by normal purine and pyrimidine bases, (ii) "stabilized" or "lost" during the first hour of postirradiation biosynthesis, (iii) to a large degree photoreversible, and (iv) not influenced by addition of enzymatically active materials extracted from *E. coli* B/r.

Because two known mutagens are producing a combined effect much greater than the total effect of the two employed separately, we termed this phenomenon "mutational synergism." The synergistic role of additional purine analogues is reported in this paper. The mutation investigated was that of *E. coli* B/r from streptomycin sensitivity to high-level streptomycin resistance.

MATERIALS AND METHODS

The methods employed for growing and starving *E. coli* B/r, for preparing and irradiating replicate populations, and for phenotypic expression, selection, and scoring of induced mutants were previously described (Shankel, 1962).

A General Electric Maxitron 250 was used for X irradiation. The dose rate in air at the locus of the cells was 1,620 r/min, as measured by a condenser r-meter (Victoreen, Cleveland, Ohio).

Viable populations were determined during the experiments by quantitatively removing

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the populations from the membranes with vigorous shaking in phosphate buffer, diluting, and plating in Brain Heart Infusion (BHI) Agar.

For the studies employing synchronized cell populations, the temperature-cycling method of Ray (1959) was employed.

The effects of the methyl purines and other reagents on the development of the radiation-induced mutants were tested by addition to the BHI or chemically defined medium employed for phenotypic expression. Preirradiation supplementation studies were done by including the chemicals in the growth medium.

The concentration of chemicals employed in these studies was 500 μg/ml, except as otherwise noted. Control experiments indicated that this concentration yielded maximal synergistic effect in all cases where synergism was obtained.

Each of the experiments reported in this paper was repeated at least three times, and yielded similar results each time. In these studies, the percentage of total mutants expressed was calculated by considering the average of the maximal number of mutants from any given treatment in the experiment to be 100%. All other data in the experiment are then expressed as percentages relative to the highest number in the experiment.

RESULTS

It is interesting to compare the relative synergistic efficiencies of caffeine, theophylline, and theobromine. In over 20 comparative experiments, the order of activity was always caffeine, theophylline, theobromine. From an initial population of 3×10^8 cells, the number of mutants resulting from ultraviolet treatment with expression on Brain Heart Infusion-caffeine (BHI-CAF) averaged 512. When expression took place on Brain Heart Infusion-theophylline (BHI-T), the average was 420, and on Brain Heart Infusion-theobromine (BHI-TB) it was 353. The average number of mutants which developed on plain BHI was 57. Preliminary studies with aminophylline indicated a synergistic effect of lesser magnitude than that obtained with theobromine. With identical population sizes (3×10^8 cells) and radiation dosages (50 ergs/mm^2), supplementation with aminophylline yielded an average of 287 high-level streptomycin-resistant mutants.

In view of the synergistic effects obtained with low ultraviolet dosages and the methylated purines, it was of interest to determine whether a similar effect might be obtained with combinations of X-ray dosages and the same purine analogues. A survival curve of the stationary-phase, glucose-starved culture of *E. coli* was determined. It was found that exposure to 3,240 r killed 44.9% of the population, whereas 12,960 r killed 98.5% of the cells. Control experiments demonstrated that the maximal numbers of mutants were phenotypically expressed in 3 hr of postirradiation incubation, regardless of the dosage employed. When cells were given varying dosages of X rays and subsequently incubated on BHI containing 500 μg/ml of theophylline or theobromine, the results indicated that no synergistic effect was obtained.

Swanson (1952) showed that ultraviolet and X-ray treatments combined, in either order, increased the mutation rate in *Aspergillus* significantly more than additively. Cheney and Rugh (1954) demonstrated that exposing *Arbacia* eggs to dosages of X rays ranging from 100 to 300,000 r, or feeding the eggs with caffeine, resulted in a delay in prophase activity. A synergistic effect between low dosages of X rays and ultraviolet light was examined with *E. coli*. Starved cultures were X-rayed with doses ranging from 3,240 to 9,720 r. Replicate populations were prepared and placed on cold phosphate-buffered agar plates. The membranes were irradiated for 3 sec with ultraviolet light and placed on prewarmed BHI agar. Control populations which received no X-ray treatment were treated similarly. The average results of a number of these experiments are shown in Fig. 1. The 100% level in this experiment represents 88 mutants; the ultraviolet treatment alone produced 66% of this number, i.e., 53 mutants. The data show that the results of the two treatments are additive rather than synergistic with the lower dosages of X rays, whereas a cancellation (probably due to killing) of the induced mutants appears when higher amounts are employed.

The experiments of Cheney and Rugh (1954) also suggested that a particular stage of cellular activity might be most susceptible to the synergistic effects of the methyl purines. Accordingly, a culture was synchronized from which a number of replicate populations were prepared. At zero time, the populations were irradiated with 50 ergs/mm^2 of ultraviolet light (except for control

populations). Two populations were immediately placed on BHI-CAF for 10 min, and were subsequently transferred to plain BHI for the remainder of the expression period. Other pairs of populations were initially placed on BHI, and were subsequently (at 5- or 10-min intervals) exposed to caffeine for 10 min and returned to BHI to complete expression. After this treatment, the mutants were selected and scored as usual. Also, at zero time, a portion of the synchronized culture was inoculated into BHI broth at 37 C; to ascertain synchrony, the population sizes were determined at the same times that the membrane populations were exposed to caffeine. The data (Table 1) show that the period from 15 to 25 min is most sensitive to the synergistic effect of the caffeine. If the cells are incubated for 30 min on BHI before exposure to the analogue, the possibility of an effect is completely lost.

The time course of "mutation fixation" in the establishment of the theophylline and theobromine effect was determined in the following manner. A number of replicate irradiated populations were prepared and placed on BHI. At intervals ranging from 10 to 60 min, pairs of membranes were transferred from BHI to BHI-T or BHI-TB, and the mutants were allowed to complete expression before being transferred to BHI-Strep for selection of the mutants. Similarly, at the same intervals, pairs of membranes initially placed on BHI-T and BHI-TB were transferred to BHI and allowed to complete expression before being transferred to the selective medium. In these experiments, a rapid decline in the number of mutants expressed occurred when the early postirradiation minutes were spent on unsupplemented BHI. This appears to be analogous to the "mutation frequency decline" observed by other authors (Witkin, 1961; Doudney and Haas, 1959). Conversely, a rapid rise in the number of mutants took place when the primary postirradiation expression was on BHI supplemented with either theophylline or theobromine. This appears similar to "mutation fixation" observed in other systems, and the results parallel the pattern observed with caffeine as the synergistic agent (Shankel, 1962).

The photoreversibility of the synergistic effects of theophylline and theobromine with both low and relatively high doses of ultraviolet

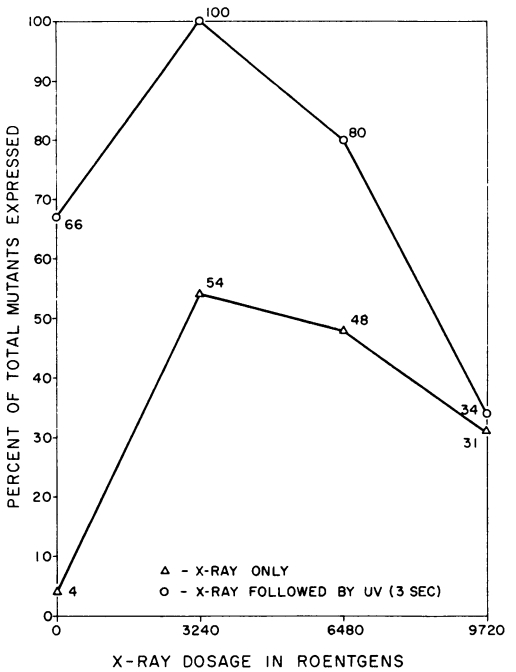

FIG. 1. *Mutants expressed after exposure to low dosages of X rays followed by low dosages of ultraviolet light.*

light was investigated. Replicate populations were irradiated in the usual manner with either a 50 or a 500 erg/mm^2 dosage of ultraviolet light. These were immediately placed on an ice bath and exposed to light from two 600-w Sylvania photoflood lamps at a distance of 10 in. for 10 min (UV-PHR). Pairs of membranes were then transferred to prewarmed BHI, BHI-T, or BHI-TB for expression, and the mutant numbers were subsequently determined in the usual manner. Control populations were wrapped in aluminum foil (ultraviolet foil) and similarly treated to determine the numbers of mutants "lost" during the time of exposure on the ice bath. The average results of a number of these experiments are shown in Table 2. Shankel (1962) previously showed that the synergistic effects of the methyl purines are obtained by supplementation into either BHI or chemically defined minimal agar, which yields similar magnitudes of effect but a lower number of total mutants. Consequently, we also determined the photoreversibility of the synergistic effect in chemically defined media, with methods identical to the previous experiments. Although total

TABLE 1. *Mutation frequency to high-level streptomycin resistance in a synchronous culture of Escherichia coli B/r exposed to a sublethal dosage of ultraviolet light followed by 10-min exposures to BHI-CAF throughout a 30-min period*

Exposure on caffeine	Population size	No. of mutants
min		
0 to 10	4.8×10^6	161
10 to 20	5.6×10^6	140
15 to 25	5.2×10^6	178
20 to 30	5.9×10^6	78
30 to 40	6.0×10^6	65
390	Synchronous population exposed to BHI	67
390	Synchronous population exposed to BHI-CAF	334
390	Spontaneous mutants on BHI	6
390	Spontaneous mutants on BHI-CAF	5

TABLE 2. *Effects of photoreversing white light on mutational synergism at varying dosages of ultraviolet light*

UV dosage	Expression medium	Population treatment	No. of mutants in 3×10^8 cells
ergs/mm²			
50	BHI-T	UV only	435
		UV-foil	426
		UV-PHR	178
	BHI-TB	UV only	392
		UV-foil	361
		UV-PHR	87
	BHI	UV only	82
		UV-foil	61
		UV-PHR	12
500	BHI-T	UV only	241
		UV-foil	234
		UV-PHR	226
	BHI-TB	UV only	222
		UV-foil	217
		UV-PHR	181
	BHI	UV only	180
		UV-foil	174
		UV-PHR	169

mutant numbers were of course lower, the pattern of the response was identical to that obtained on the complex BHI media. This was true for both ultraviolet dosages.

Supplementing the preirradiation growth medium with caffeine failed to produce any synergistic effect with subsequent ultraviolet treatment (Shankel, 1962). This was also the case with theophylline and theobromine; i.e., the analogues had to be present in the post-irradiation medium to evince their effect. Growing the cells in their presence did not result in any observable effect.

Wyss et al. (1948) showed that catalase may negate some of the "indirect" effects of ultraviolet radiation. An experiment was performed to determine whether supplementation of the pre- and postirradiation media with catalase and peroxidase would alter the mutation frequency to high-level streptomycin resistance. Replicate populations were prepared as usual, except that the growth medium contained 5 mg per liter of catalase and peroxidase. The populations were irradiated with 50 to 60 ergs/mm² of ultraviolet light, immediately transferred to BHI or BHI-CAF supplemented with 5 mg per liter of catalase and peroxidase, and incubated until complete phenotypic expression. The results indicated that, in this system, catalase and peroxidase did not alter mutation frequency when the cells were expressed on enzyme-supplemented BHI and, furthermore, the peroxide-destroying enzymes did not alter the mutational synergism produced by ultraviolet light and caffeine.

The synergistic effects of theophylline and theobromine with various dosages of ultraviolet light were investigated. Replicate populations were prepared and exposed to doses of ultraviolet light ranging from 3 (50 to 60 ergs/mm²) to 30 sec (500 to 600 ergs/mm²), then immediately transferred to either BHI, BHI-T, or BHI-TB, and incubated for the time required for maximal expression of the mutants. The mutants were selected, and the numbers were determined, in the usual manner. The results of these experiments indicated that only relatively low doses of ultraviolet light act together with theophylline or theobromine to produce the synergistic mutational effect. The highest degree of synergism was obtained with a dosage of 50 ergs/mm²; synergism was completely absent when a dosage of 450 ergs/mm² or higher was employed.

DISCUSSION

Certain purine analogues acting in conjunction with very small amounts of ultraviolet light produce tremendous increases in mutant

numbers. Other investigators (Lieb, 1961; Witkin, 1959), employing other mutagenic systems and higher dosages of ultraviolet light also showed the existence of the synergistic type of mutational effect produced by combinations of ultraviolet light and caffeine or theophylline. The present report, however, is the first demonstration that theobromine demonstrates a similar ability. With three chemical agents able to produce the synergistic effect, a comparison of their relative efficiencies becomes possible. All three were employed in concentrations of 500 μg/ml (2.57 mM caffeine, 2.77 mM theophylline and theobromine), previously shown to be the lowest concentration yielding a maximal effect. Caffeine demonstrated a significantly higher activity, suggesting that the extra methyl group possessed by caffeine increases its activity. Further, because theophylline demonstrated greater activity than theobromine, in spite of the fact that molar concentrations were identical and that the two molecules differ only in location of their two methyl groups, it appears that specific structure must influence the degree of synergistic activity displayed by these compounds. The chemical groups and their specific locations essential to the activity of these related compounds must next be determined.

The observation by Swanson (1952) that combinations of ultraviolet and X-ray treatment produced mutation rates in *Aspergillus* higher than would be expected on an additive basis contrasts with the observations of Kaufman and Hollaender (1946) and of Swanson (1944) that ultraviolet treatment decreased the chromosomal aberrations resulting from X-ray treatment of *Tradescantia* spp. and *Drosophila* spp. The present results indicate that when sufficiently low dosages of X rays and ultraviolet light are employed, in this system at least, there is neither a synergistic effect between the two nor a decrease in mutation frequency, but simply an additivity of effects. This disappears when the dosage of one of the agents is increased.

It is interesting to note that no indication of a synergistic mutational relationship between X rays and methyl purines was observed. This appears to rule out the involvement of any of the suggested overlapping mechanisms of action of X rays and ultraviolet light, and supports the suggestion (Cheney and Rugh, 1954) that, even though exposure of *Arbacia* eggs to either X rays or caffeine tended to produce a delay in the onset of prophase activity, the basic mechanisms of action of these two agents appeared to be different.

The results obtained with synchronized cultures indicate that the highest number of mutants obtained from a 10-min treatment with caffeine resulted when that treatment came 15 to 25 min after the irradiation. This could reflect either an effect on a particular stage of nuclear division, or an inhibition or stimulation of the development of a particular enzyme or enzyme system.

The results in which the time course of "mutation fixation" and the time required for "mutation frequency decline" were determined for theophylline and theobromine confirm earlier observations made with caffeine (Shankel, 1962).

The studies on photoreversibility lead to some interesting observations. When low dosages of ultraviolet light are employed, a very large percentage of both the ultraviolet-induced mutants and the synergistically produced mutants can be photoreversed or "photoprevented." However, when higher lethal dosages are employed, the degree of photoreversibility is greatly decreased. This supports an earlier suggestion (Shankel and Wyss, 1961) that, when mutations are induced by nonlethal dosages, at least two mechanisms of ultraviolet action are involved, and that the role of one (or more) of these mechanisms is decreased in significance as the radiation dosage is increased.

The demonstration that no synergistic effects from the addition of theophylline or theobromine are obtained when higher dosages of ultraviolet light are employed confirms earlier observations with caffeine (Shankel, 1962), and provides further evidence for the uniqueness of the mechanism of action of nonlethal dosages of ultraviolet light.

It is interesting to speculate upon the mechanism of action of the methyl purines in creating their synergistic effect with low dosages of ultraviolet light. Koch (1956) showed that only a very low level of incorporation of caffeine into the deoxyribonucleic acid of normal *E. coli* occurs. It is possible that prior exposure to ultraviolet light alters the amount of caffeine incorporated, but preliminary results in our own laboratory indicate that this is not the case. Consequently,

it would appear that this synergistic effect is not due to caffeine replacement of a normal purine base in the genetic material.

Lieb (1961) suggested that ultraviolet light induces formation of a demethylating enzyme that removes the methyl group from the 7 position of caffeine, permitting the formation of a stable caffeine deoxyriboside. Since guanosine and adenosine do not antagonize the caffeine effect, and since theophylline, which is unsubstituted in position 7, produces a lower degree of synergism than does caffeine, this mechanism would not seem to be involved in the synergism.

It is well known that ultraviolet light produces photoproducts whose presence during nucleic acid synthesis may result in copy errors. Known photoproducts which can be incorporated into nucleic acid include the hydration products of cytosine and uracil (Sinsheimer, 1954, 1957) and a dimer of thymine (Beukers and Berends, 1960; Marmur et al., 1961). Lieb (1961) suggested that irradiated bacteria have available a "dark recovery" system, probably enzymatic, which removes or alters photoproducts, resulting in the reduction of the probability of mutation during nucleic acid synthesis. This could be similar to the "mutation frequency decline" proposed by Doudney and Haas (1959). Lieb suggested that the caffeine either competes with the photoproducts for the "dark recovery" system or combines with nucleic acid [DNA, ribonucleic acid (RNA), or both] and prevents access of the "dark recovery" system to the photoproducts while they are acting. In view of the observation by Witkin (1961) that acriflavine (which combines with DNA and RNA) has an effect similar to that of caffeine on an auxotrophic to prototrophic mutation, and in view of the present observations on photoreversibility of the potential analogue effect, the second suggestion seems more tenable. At this time, however, the actual mechanism by which the methylated purines bring about their drastic changes in mutation frequency remains obscure.

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