# "MUTATIONAL SYNERGISM" OF ULTRAVIOLET LIGHT AND CAFFEINE IN *ESCHERICHIA COLI*

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ABSTRACT

SHANKEL, DELBERT M. (University of Kansas, Lawrence). "Mutational synergism" of ultraviolet light and caffeine in *Escherichia coli*. J. Bacteriol. **84**:410–415. 1962.—When ultraviolet-induced mutations of *Escherichia coli* B/r develop in the presence of caffeine, there is a large increase in mutant numbers. The development of these "extra" mutants is not affected by the presence of normally occurring purines and pyrimidines, azauracil, azathymine, or 5-methyltryptophan, but many of the mutants can be photoreversed. They are "stabilized" or "lost" during the first hour of postirradiation growth. Enzymatically active materials extracted from *E. coli* cells do not interfere with the caffeine effect, and variation of the growth temperature during the development of the mutations fails to alter the effect. "Prefeeding" the cells with caffeine prior to irradiation does not produce the response. The effect is observable with both stationary-phase and log-phase populations, but is not observable when a relatively high dosage of ultraviolet light is employed.

When *Escherichia coli* B/r is subjected to nonlethal amounts of ultraviolet light, significant increases in mutation frequency may still be observed (Matney, Shankel, and Wyss, 1958). This system provides distinct advantages for studying effects of pre- and postirradiation treatments on the development of induced mutations, since no corrections for cell death are involved and the effects of the treatment are restricted to effects on mutational development.

Novick and Szilard (1951) induced mutants in their chemostat by treating cells with the purine analogue, caffeine, and Witkin (1958) observed a many-fold increase in the number of reversions from auxotrophy to prototrophy when ultraviolet-induced mutants were allowed to become phenotypically expressed in a caffeine-containing medium. This phenomenon was also observed in our laboratory during a search for chemicals which would affect the development of ultraviolet-induced mutations to high-level streptomycin resistance (Shankel, 1961). Lieb (1961) reported studies on the combined effects of caffeine and ultraviolet employing an auxotrophic to prototrophic mutation. This paper is a report of further studies on this phenomenon of "mutational synergism" between two apparently unrelated mutagenic agents, caffeine and ultraviolet light, employing a mutation from streptomycin sensitivity to high-level streptomycin resistance.

## MATERIALS AND METHODS

*E. coli* B/r was maintained and transferred biweekly on slants of a chemically defined medium found to favor a smooth-colony type. For irradiation studies, a starved culture was prepared by inoculating a minimal broth containing glucose in the growth-limiting concentration of 0.02% and incubating with shaking at 36 C for 14 to 18 hr. The culture attained its maximal growth of approximately $3 \times 10^8$ cells/ml in 8 hr, and the additional time was allowed for the depletion of endogenous nutrients. After two washings with cold phosphate buffer (pH 7.0), the organisms were resuspended in sufficient phosphate buffer to give a density of $3 \times 10^8$ cells/ml and incubated with shaking for an additional 2 hr at 36 C for more complete exhaustion of the endogenous nutrients. Replicate populations of starved stationary-phase cells were prepared by impinging samples of the microbial suspension on membrane filters. The 5.0-cm "Bacti-Flex" membranes were obtained from Carl Schleicher and Schuell Co. of Keene, N.H. The membranes were then placed on cold phosphate-buffered agar plates for exposure to the desired amount of irradiation.

The ultraviolet treatments were performed with a 15-w G. E. germicidal lamp. In all experiments, except as otherwise noted, the cells

were exposed to 50 ergs/mm², which is a nonlethal dosage for these cells under these conditions. The ultraviolet treatments and all subsequent operations were carried out under yellow light to minimize photoreversal of induced mutations.

After irradiation the membranes were transferred to prewarmed (37 C) plates of the medium employed for phenotypic expression and incubated for the period of time which preliminary control experiments had shown to be required for maximal expression of the mutants. The M-9 chemically defined medium as modified by Haas and Doudney (1957), the complete expression medium, Difco Brain Heart Infusion (BHI), and all media containing supplements were prepared with only 0.75% agar. These soft-agar media were firm enough to support the membranes and yielded better growth than plates containing 1.5% agar.

After the time required for expression of the mutant phenotype had elapsed, the membranes were transferred to soft BHI agar plates containing 1 mg/ml of dihydrostreptomycin sulfate (BHI-Strep) for selection of the mutants in the positionally fixed populations. At the same time, control populations (e.g., nonirradiated to determine the spontaneous level) were also transferred to the streptomycin medium. After an additional 48 to 72 hr of incubation (37 C) the number of high-level streptomycin-resistant mutants resulting from a given treatment was determined by scoring the number of colonies which developed in the presence of the drug.

The effects of caffeine and other chemicals on the development of the radiation-induced mutants were tested by addition to the BHI or chemically defined medium employed for phenotypic expression. Each experimental result reported is the average of several experiments, in each of which the membrane populations were employed in duplicate or triplicate.

## RESULTS

Table 1 presents the basic data on the effect of caffeine on the development of mutations to streptomycin resistance. When replicate populations were treated as described above and incubated on soft BHI agar containing 500 μg/ml of caffeine (BHI-CAF) for expression, a tenfold increase in the number of mutations to streptomycin resistance was observed. Since only about one-third of those mutations produced by nonlethal amounts of ultraviolet, which develop on BHI, are able to be expressed on chemically defined medium (Shankel and Wyss, 1961), the effect of caffeine supplementation into minimal medium was also determined. In this case an eight-to tenfold increase in the number of mutants was also observed. Furthermore, caffeine employed alone, without prior ultraviolet treatment of the cells, produced no increase in mutation frequency above the spontaneous level. (The effect was observable only when UV and caffeine were employed together.)

TABLE 1. *Effect of expression medium on development of mutations induced by nonlethal dosages of ultraviolet light and on "spontaneous" mutation frequency*

| Postirradiation expression medium | Number of mutants expressed |
|---|---|
| BHI | 53 |
| Modified M-9 minimal | 36 |
| Minimal* | 255 |
| BHI* | 568 |
| *Unirradiated control populations* | |
| Minimal | 4 |
| Minimal* | 4 |
| BHI | 6 |
| BHI* | 7 |

* Plus 500 μg/ml of caffeine.

A number of other purine and pyrimidine analogues were used to determine whether any of them might produce similar results. Some results, typical of those obtained with other analogues, are shown in Table 2. They indicate that only theophylline and theobromine, which bear definite structural relationships to caffeine, produced effects similar to those produced by caffeine.

Further studies were performed employing caffeine. The concentration of caffeine producing the greatest number of "extra" mutants was determined to be 500 μg/ml by utilizing varying concentrations in the BHI expression medium.

In addition, since the effect was first observed with a starved stationary-phase population, the effect of the caffeine was determined using a log-phase culture, with all other parameters of the experimental method remaining unchanged. The mutationally synergistic effect of the caffeine was also produced in this system.

To obtain information concerning a possible

TABLE 2. *Effects of purine and pyrimidine analogues, other than caffeine, on the development of mutations induced by nonlethal dosages of ultraviolet light*

| Analogue employed | Concn | Mutants* |
|---|---|---|
| | μg/ml | % |
| 8-Azaguanine | 200 | 100 |
| 6-Azathymine | 1,000 | 150 |
| Thiouracil | 500 | 100 |
| 6-Mercaptopurine | 500 | 90 |
| 5-Bromouracil | 1,000 | 70 |
| 5-Bromouracil | 500 | 100 |
| 5-Nitrouracil | 500 | 90 |
| 4-Aminopyrazolo (3,4d) pyrimidine | 500 | 110 |
| Theophylline | 500 | 810 |
| Theobromine | 500 | 820 |

* Number of mutants on BHI controls is used as 100% level.

site of action of the caffeine, the cells were "prefed" with caffeine by incubating the cells for 1.5 to 2 hr in M-9 medium to which 500 μg/ml of caffeine were added. Replicate populations were then prepared in the usual manner and subjected to the standard ultraviolet dosage. No increase in mutant numbers above the control level (no caffeine treatment) was observed. "Prefeeding" the cells with caffeine failed to alter the mutagenesis normally attributable to the ultraviolet light.

The effect of a high ultraviolet dosage on the caffeine effect was investigated in the following manner. Standard cell populations were exposed to the usual dosage of 50 ergs/mm² and also to a relatively high dosage of 1,350 ergs/mm². Subsequently, the mutations were allowed to develop on BHI or BHI-CAF in the usual manner for the period of time shown by control experiments to yield maximal expression. The mutants were then selected on BHI-Strep as usual. The populations receiving the nonlethal dosage gave the expected response (i.e., a tenfold increase by the caffeine), but the populations receiving the high dosage showed no effect attributable to the presence of the caffeine (i.e., no increase in numbers of mutations).

An attempt was made to reverse the caffeine effect competitively by the inclusion of normally occurring purine and pyrimidine bases and their ribotides with caffeine in the BHI expression medium. As Table 3 shows, the inclusion of these

TABLE 3. *Effects produced by supplementing various normal bases and an enzyme preparation into postirradiation BHI-CAF expression medium**

| Expression medium | Mutant numbers |
|---|---|
| BHI only | 66 |
| BHI-CAF | 500+ |
| BHI + guanosine | 67 |
| BHI-CAF + guanosine | 500+ |
| BHI + adenine | 87 |
| BHI-CAF + adenine | 500+ |
| BHI + adenosine | 63 |
| BHI-CAF + adenosine | 500+ |
| BHI + guanine | 65 |
| BHI-CAF + guanine | 500+ |
| BHI-CAF + adenine + guanine + thymine + cytosine | 500+ |
| *Enzyme experiment*† | |
| BHI only | 18 |
| BHI + enzyme preparation | 23 |
| BHI-CAF | 194 |
| BHI-CAF + enzyme preparation | 193 |

* All concentrations shown were 500 μg/ml.

† Smaller initial populations (ca. $1 \times 10^8$) were used in this series of experiments.

compounds, singly or in combinations and over a wide range of concentrations, failed to alter the increase in mutagenesis attributable to the caffeine. In addition, since it had been suggested (Witkin, 1958) that caffeine might act by interfering with the enzymes of deoxyribonucleic acid (DNA) synthesis, *E. coli* B/r was grown in BHI broth and 40 g of cells (wet wt) were harvested. These cells were mechanically disrupted in a Braun shaker; the cell fragments were removed by centrifugation and filtration in the cold. (The mechanical disrupter was purchased from Braun Apparatebau, Melsungen, Germany.) The filtrate, which possessed methylene blue reductase activity, was incorporated into BHI-CAF expression medium. This also failed to alter the number of mutants from the number obtained on BHI-CAF alone.

A study was made of the photoreversibility of the "extra" mutants resulting from the presence of caffeine. Cells on membrane filters were ultraviolet irradiated in the usual manner. These were then immediately placed on an ice bath and exposed to light from two 300-w photoflood lamps at a distance of 10 in. for a period of 10 min. Pairs of membranes were then transferred to prewarmed BHI agar and BHI-CAF agar for

expression, and the number of mutants subsequently determined. Control preparations were wrapped in aluminum foil and similarly treated to determine the numbers of mutants "lost" during the time of cold treatment in the absence of white light (Fig. 1). A few mutants, on both BHI and BHI-CAF media, were "lost" in the absence of white light but, in addition, in each case about two-thirds of the eventual numbers of mutants could be photoreversed.

Doudney and Haas (1958) and others have demonstrated a sensitive period following ultraviolet irradiation termed "mutation stabilization" during which the eventual yield of mutations can be altered by the postirradiation environment. This observation has been confirmed for mutations induced by nonlethal amounts of ultraviolet light (Shankel and Wyss, 1961). The period required for "stabilization" of the caffeine effect was determined in the following manner. Membranes were irradiated and placed on BHI medium. At intervals ranging from 15 min to 3.5 hr (complete expression), pairs of populations were transferred to BHI-CAF to complete their expression. Conversely, pairs of replicate membranes were initially placed on BHI-CAF and then transferred to plain BHI at intervals from 15 min to 3.5 hr to determine the "mutation frequency decline" (Doudney and Hass, 1958).

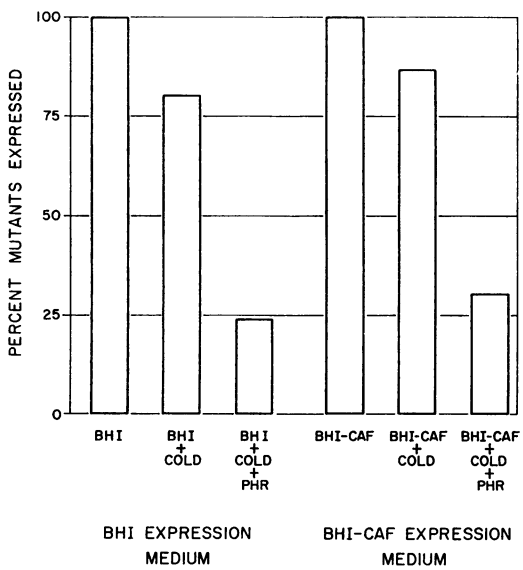


FIG. 1. *Effects of cold temperature and photoreversing light on mutational synergism. The development of ultraviolet-induced and ultraviolet-caffeine-induced mutations when the irradiation is followed immediately by brief treatments with cold or cold plus white light, prior to exposure of the cells to the medium used for phenotypic expression. The total numbers of mutants produced in control populations on BHI and on BHI-CAF are indicated as 100% in each case.*

As these data show, the caffeine must be present during the first hour of postirradiation incubation to exert a significant effect; any delay in placing the cells in the presence of caffeine results in a decrease in the eventual number of mutants which is directly related to the length of the delay (Fig. 2).

To obtain evidence concerning the possible metabolic processes involved in the caffeine effect, a number of analogues and antibiotics were employed. Membranes were prepared, irradiated, and placed on minimal agar, minimal agar plus caffeine, or minimal agar plus caffeine plus analogue for the time shown to be required for stabilization. Subsequently, they were transferred to minimal agar to complete expression and finally selected and scored on BHI-Streptomycin. Azauracil, azathymine, or 5-methyltryptophan in concentrations of 20 $\mu$g/ml did not retard the development of the mutations. Treatment with 4 $\mu$g/ml of chloramphenicol effectively suppressed the development of the mutations, as did treatment with 10 $\mu$g/ml of puromycin.

The effect of temperature upon the joint mutagenic action of ultraviolet light and caffeine was tested in the following manner. Replicate populations, prepared and irradiated as usual,

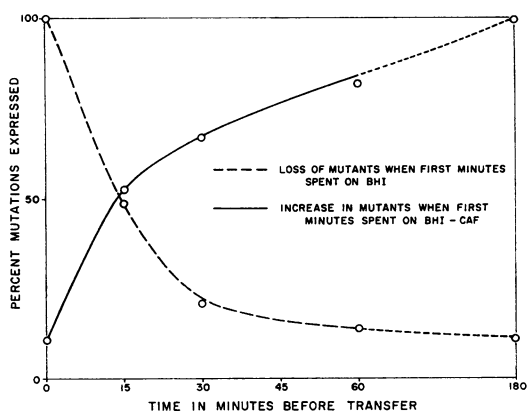


FIG. 2. *"Mutation stabilization" and "mutation frequency decline." Mutants are rapidly "lost" in the absence of caffeine, and rapidly "stabilized" in the presence of the analogue.*

TABLE 4. *Relationships of expression and selection temperatures to the development of mutants produced by the combined action of ultraviolet light and caffeine*

| Expression | | | Selection temperature | Mutant numbers |
|---|---|---|---|---|
| Medium | Time | Temperature | | |
| | *hr* | *C* | *C* | |
| BHI | 3.5 | 37 | 37 | 70 |
| BHI-CAF | 3.5 | 37 | 37 | 335 |
| BHI | 5 | 30 | 37 | 64 |
| BHI-CAF | 5 | 30 | 37 | 130 |
| BHI | 7 | 30 | 37 | 76 |
| BHI-CAF | 7 | 30 | 37 | 304 |
| BHI | 5 | 30 | 30 | 46 |
| BHI-CAF | 5 | 30 | 30 | 136 |
| BHI | 7 | 30 | 30 | 39 |
| BHI-CAF | 7 | 30 | 30 | 356 |

were placed on BHI or BHI-CAF at 30 and 37 C for periods shown by control experiments to yield maximal numbers of mutants. Some of the populations which had undergone phenotypic expression at 30 C were transferred to BHI-Strep at 37 C for selection of mutants; others were transferred to BHI-Strep at 30 C for selection. The control populations, which had been phenotypically expressed at 37 C, were also selected at 37 C, as usual. The results in Table 4 indicate that altering the postirradiation temperature during the phenotypic-expression period does not alter the relative effect of caffeine on the development of the induced mutations. When the total process, expression and selection, takes place at 30 C, the caffeine produces the usual tenfold increase.

## DISCUSSION

It is tempting to propose that the caffeine is incorporated into the DNA, resulting in a change in base sequence and thus producing the increased number of mutants. However, Koch (1956) demonstrated that only minute amounts of radioactive caffeine were incorporated into the DNA of *E. coli* B/1t. He also observed that the presence of caffeine in a concentration of 1,630 μg/ml did not interfere with the growth rate of the organism. Since, however, we are employing a starved-culture system and subjecting the cells to ultraviolet light, we feel that the possibility of incorporation should not be completely eliminated at this time. It is conceivable that these differences in cell treatment might alter the incorporation of the analogue.

Greer (1958) observed that 250 μg/ml had on effect on the growth rate or the spontaneous mutation rate from auxotrophy to prototrophy in *E. coli*. Witkin (1958), however, postulated that the increased mutagenesis was due to an inhibition of DNA synthesis by caffeine, allowing more time for "promutants" to become mutants before the start of DNA synthesis. This seems unlikely in our system, since other purine and pyrimidine analogues failed to create this effect and since a lowering of the temperature to 30 C during the caffeine treatment failed to alter the enhancing effect of the caffeine on the development of the mutations.

Lieb (1961) made many observations similar to those presented here on an auxotrophic to prototrophic mutation, and suggested that caffeine interferes with a "dark repair" enzyme system which removes ultraviolet photoproducts whose presence during DNA synthesis leads to mutations. The results presented here tend to support, to some degree, this hypothesis. Alternatively, however, it also seems possible that the caffeine might exert its synergistic effect on ultraviolet-induced mutations by potentiating in some manner the ultraviolet photoproducts and allowing them a longer period of time in which to act.

Merz, Swanson, and Cohn (1961) have demonstrated that 8-ethoxycaffeine and other radiomimetic compounds cause breaks in the chromosomes of the roots of *Vicia faba*. This indicates the possibility that, in the present case, a genetic instability might be initiated by the ultraviolet and further chromosome damage brought about by the subsequent caffeine treatment. If this is the case, then it is conceivable that resulting changes in base sequence could produce the observed mutagenic effect.

At the present time, no definitive conclusion can be reached concerning this mutational synergism between caffeine and ultraviolet light. Further studies are now in progress in an attempt to determine the mechanism and site of action involved in this biological effect.

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