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Mutagenesis and Mutant Selection in *Physarum polycephalum*

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Summary. *Physarum polycephalum* myxamoebae were exposed to ultraviolet irradiation and plated in the absence and presence of caffeine. Caffeine reduces the shoulder on the UV[1] dose-survival curve, thereby increasing the UV-sensitivity for survival. Caffeine alone is a moderate mutagen. Used in conjunction with UV a strong mutagenic action is observed. Active growth is required for both of these mutagenic actions.

Populations of *Physarum* myxamoebae mutagenized with NMG or EMS could be enriched for two classes of mutants by incubating at high temperature (30° C) with 5-bromodeoxy-uridine-substituted bacteria followed by irradiation with long wave UV light and recovery at low temperature (23° C). One class of mutants was obtained in high yields after repeated cycles of light inactivation. These are not heat sensitive. Rather they are defective in utilization of DNA precursors provided by the bacteria. The other mutant class, obtained in low yields after limited selection, are heat sensitive. Three independent mutants of this kind, all eaky, were obtained. Reconstruction experiments show that all are selectants.

Introduction

The study of eukaryotic control mechanisms is facilitated in the myxomycete *Physarum polycephalum* where the diploid phase (the plasmodium) shows spontaneous synchrony (Rusch, 1970). The haploid phase (the myxamoebae) is generated by sporulation of the plasmodium. Mating, followed by zygote and plasmodium formation, occurs between cells with different mating types (Dee, 1966a). Thus genetic analysis is possible in *Physarum*.

These features make the organism suited for analysis of control mechanisms by genetic and biochemical means. In order to develop further this organism, we have explored methods of mutagenesis and mutant selection in *Physarum*. Successful mutagenesis was achieved using UV-irradiation followed by incubation in presence of caffeine, a presumed inhibitor of repair (Sauerbier, 1964).

In selecting for mutants, our goal has been to obtain mutants with heat sensitive DNA synthesis. Toward this end, we have utilized the ability of BUdR to substitute for TdR in the DNA of growing cells, thereby making the cells light-sensitive. This "suicide"-selection has been valuable in obtaining such mutants in bacteria (see, for example, Carl, 1970).

Methods

1. Strains

Myxamoeba strain RSD4 was used throughout. For description, see Haugli (1971). *Escherichia coli* strain KB was used as food for the amoebae. For incorporation of BUdR, or

1 Abbreviations: UV, ultraviolet irradiation; NMG, N-methyl-N′-nitro-N-nitroso-guanidine; EMS, ethyl-methane-sulfate; BUdR, 5-bromo-deoxy-uridine; FUdR, 5-fluoro-deoxyuridine; TdR, thymidine.

radioactive TdR, *E. coli* K12 strain CR34 *(thy⁻ leu⁻ thr⁻ B_1^-)* was used. For preparation, see Haugli (1971).

2. *Myxamoebae Culture Methods*

Myxamoebae were grown at 25°C and 85% relative humidity. When selecting for heat sensitive mutants, the permissive temperature was 23°C and the restrictive temperature was 30°C. Agar plates were prepared with 2% bactoagar and 0.05% liver infusion (Oxiod). Cells were spread on the plates together with 10^{10} formalin-inactivated bacteria. For preparation of plates and bacteria, see Haugli (1971).

Growth experiments and preparation of large amounts of log-phase cells were done by inoculating 5×10^4 cells per plate and allowing three days for growth at 25°C. Cloning was done by picking cells with a sterile toothpick from single plaques. Details are described elsewhere (Haugli, 1971).

3. *Mutagenesis*

Mutagenesis was always performed on the haploid myxamoebae in these experiments. The UV-source was a General Electric 15 W germicidal lamp. Cells were plated after UV-irradiation on plates with or without 0.025% caffeine. Details of irradiation conditions and screening techniques are described elsewhere (Haugli, 1971).

NMG treatment was at 10–40 μg/ml of NMG freshly dissolved in 0.05 M phosphate buffer pH 7.0, for 30 minutes.

EMS was used at 0.2 M in the same buffer, also for 30 minutes. 24 hours of post treatment growth was used to allow fixation and expression at mutations.

4. *Screening and Selection for Mutants*

Experiments involving UV- and caffeine-mutagenesis used plaque size, and (occasionally) cycloheximide resistance as markers. The former trait was suggested by studies in *Dictyostelium* (Loomis, 1968); the latter by previous work in *Physarum* (Dee, 1966b).

Identification of plaque-size mutants involved screening of primary plates, formed by plating about 100 mutagenized cells per plate. Small plaques were cloned 2–3 times and plaque diameters were compared with a wild type control. Some experiments involved an analysis of induction of plaque size mutants in small clones rather than large populations. In these cases colonies were plated individually and quantitatively after the mutagenic treatment. For details, see Haugli (1971).

Selection for cycloheximide resistant mutants was done on plates containing cycloheximide at 4 μg/ml, as described by Dee (1966b).

Indirect selection for heat sensitive mutants was followed by screening. The following procedure was used. The DNA of *E. coli* K12 CR34 *thy⁻ leu⁻ thr⁻ B_1^-* was substituted about 50% with BUdR for TdR and these bacteria were formalin-inactivated and fed to myxamoebae. The myxamoebae, growing at 30°C on these bacteria, became light-sensitized and could be killed approximately 3 decades after a 2–4 fold increase, giving an enrichment of 500–250 fold for those cells that for any reason could not incorporate BUdR from bacterial DNA at 30°C. The rationale was to enrich for mutants with heat sensitive DNA synthesis this way. Repeated cycles of selection were expected to give greater enrichment for heat sensitive mutants. However, in the present work, extensive selection resulted in populations of mutant myxamoebae unable to utilize BUdR at any temperature. Thus, limited selection followed by extensive screening had to be used. This screening, then, involved comparison of growth of clones at 23°C and 30°C.

5. *Statistical Analysis*

The confidence limits of the mutant frequencies are estimated from the sampling error expected for a Poisson distribution. If a sample of size N (cells) contains a small number of mutants, n, then the sampling error of n is $\pm\sqrt{n}$. Thus the mutant frequency is $(n \pm \sqrt{n})/(N)$. 80% confidence limits on results were calculated according to Student's t-test.

Results

A. Lethal and Mutagenic Action of Ultraviolet Irradiation and Caffeine

1. Lethal Effect of UV and Caffeine

The mutagenic and lethal effects of UV-irradiation in a number of systems are well known. It is also known that the damage caused by UV is subject to repair in a number of cell types (see, for example, Witkin, 1969; Rauth, 1967).

Furthermore, it has been shown that xanthine derivatives such as caffeine can interfere with the repair process, thereby affecting the lethal and mutagenic effects of UV irradiation (see, for example, Sideropoulos and Shankel, 1968; Clarke, 1968; Rauth, 1967). In view of previous failures to induce mutants in *Physarum* by UV or other means we have investigated the effects of UV and caffeine in this organism.

Preliminary experiments performed by Mr. David Kuter showed that the growth rate of myxamoebae decreased at caffeine concentrations between 0.025% to 0.125%, while levels beyond 0.125% completely abolished growth. When an exponential culture of myxamoebae was harvested, irradiated in water for various times at a dose rate of 4.2 ergs mm^{-2} sec^{-1}, and then plated on normal plates and on plates containing 0.025% caffeine, the results shown in Fig. 1 were obtained. The extensive shoulder in the inactivation curve is greatly reduced by caffeine, indicating sensitization to UV light. Only postirradiation treatment is effective: no sensitization was observed when caffeine was present only prior to or during irradiation.

2. Mutagenic Effect of UV

For these studies "plaque-size" was used as marker. Fig. 2A shows growth curves of irradiated and untreated control-cultures. A slight depression of growth rate with quick recovery is evident in the irradiated cultures.

Fig. 2B shows the kinetics of appearance of plaque-size mutants after irradiation. The frequency rises from a spontaneous level of 0.2×10^{-2}, as measured in an untreated culture at the time of irradiation, to 2.6×10^{-2} nineteen hours after exposure. This rise is followed by a slow decline in mutant frequency, presumably because the mutant cells are outgrown by wild type cells.

It can be concluded that after this dose of UV the appearance of plaque-size mutants is a function of time, or of growth, or both. If a plate culture of myxomoebae is harvested and then replated, a growth lag is induced (Haugli, 1971). Fig. 2A, curve IV, shows the growth curve of a culture that was irradiated at the time of replating. Fig. 2B, curve II, shows the induction of plaque size mutants in this culture. It is seen that the frequency of plaque size mutants never rises above 0.18×10^{-2}. Since the log-phase and lag-phase cultures received the same dose of UV, their potential to produce mutants must initially have been the same. We conclude therefore, that the growth-lag which occurs in the lag-phase cultures erases this potential.

3. Mutagenic Effect of Caffeine

When cells were grown on 0.025% caffeine, sampled and analysed at intervals for mutant frequency, it was found that the frequency of plaque-size mutants rose during the first 24 hours, the lag-phase, from 0.1×10^{-2} to 0.8×10^{-2}. During

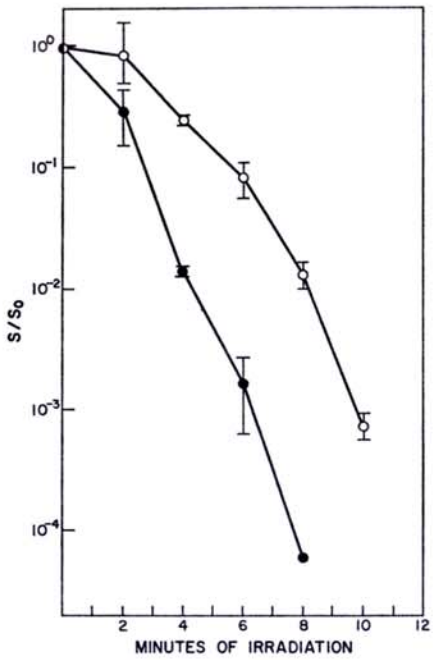

Fig. 1. UV survival curves for cells titered on normal medium (○) and on caffeine medium (●). 5 ml of cell suspension (myxamoebae, strain RSD4) at 5×10^6 per ml, was irradiated in a 10 cm petri dish at a dose rate of 4.2 ergs mm^{-2} sec^{-1}. Titering for survival was performed on normal medium (○) or on plates supplemented with 0.025% caffeine (●). The 80% confidence limits were calculated according to Student's *t*-test. Abscissa: UV dose, in minutes of irradiation. Ordinate: Survival, S, on a logarithmic scale, normalized to initial titer on normal plates, S_0

the next 48 hours, the log-phase, the mutant frequency increased to 6.0×10^{-2}. Thus caffeine alone has a substantial mutagenic effect in this system, and the mutagenicity is enhanced by active growth.

4. Mutagenic Effect of UV in Presence of Caffeine

Fig. 3A shows the effect of UV and caffeine on the growth curve of log-phase cultures (curve III), and Fig. 3B shows the kinetics of appearance of plaque-size mutants (curve I). The spontaneous mutant level of 0.2×10^{-2} increases 125-fold to 25.5×10^{-2}. This initial increase among the 10% survivors is followed by a sharp decrease in mutant frequency upon further growth with caffeine.

If the same experiment is done on lag-phase cells, Fig. 3B, curve II, a smaller increase in mutant frequency is observed. The curves in Fig. 3 shows that caffeine works synergistically with UV to increase the mutagenic effect of irradiation. They also show that active growth is necessary for optimal mutagenic action.

When the integral UV dose was raised from 50 ergs mm^{-2}, in the above experiment, to 125 and then to 250 ergs mm^{-2}, the mutant frequency in log phase

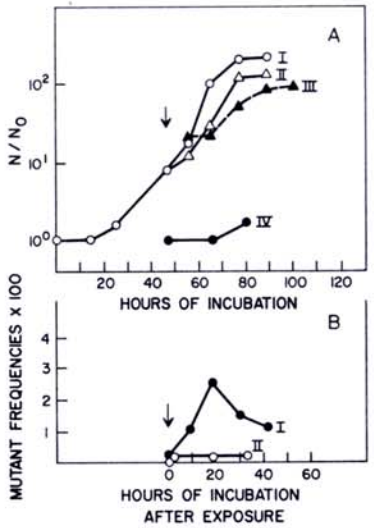

Fig. 2. A, Growth curves after UV irradiation of log-phase and lag-phase cultures. Cells were grown on Millipore with liver infusion medium. One set of cultures was left untreated to serve as control (○), curve *I*. At 46 hours the 5×10^4 cells inoculated per culture had increased about 10-fold. At this time (arrow) one set of cultures was irradiated with 6 seconds of UV at dose rate 8.5 ergs mm^{-2} sec^{-1} (△), curve *II*. (In the same experiment a third set of cultures was transferred to caffeine medium and irradiated with the same dose of UV, (▲), curve *III*. See Fig. 3.) Curve *IV* (●) is the growth curve for cultures irradiated at the time of plating—at the beginning of lag-phase. Abscissa: Hours of incubation at 25°C. Ordinate: Cell yield (N) normalized to inoculum size at time zero, N_0, on a logarithmic scale. B, Kinetics of appearance of mutants after UV irradiation of log-phase and lag-phase cells. Cells were irradiated as in Fig. 2A. At the time intervals indicated, cells were harvested and plated for screening of plaque-size mutants. Curve *I* (●), log-phase cells. Curve *II* (○), lag-phase cells. Abscissa: hours of incubation at 25°C. Ordinate: mutant frequencies × 100

cultures did not increase, and in the latter case was actually significantly decreased. We attribute this to the increased growth lag caused by higher doses of UV.

5. Clonal Analysis of Induction of Plaque-Size Mutants

Untreated cultures of myxamoebae contain plaque-size mutants at a frequency of $0.1–0.2 \times 10^{-2}$. Growth on caffeine increases this frequency to 6×10^{-2}. Finally, UV irradiation followed by growth on caffeine increases the mutant frequency nearly 200-fold to 25.5×10^{-2}. That this increase reflects induction of mutants rather than selection of pre-existing mutants is indicated by analysis of clones. Cells were plated, 80 at a time, on (a) water agar (b) 0.025% caffeine agar and (c) plates containing 20 ml of regular growth medium.

(a) Spontaneous Rate. The cells plated on water agar were screened after 7 days at 25°C. Of the 1863 plaques inspected, 2 mutants were found, giving a spontaneous frequency of $(0.1 \pm 0.08) \times 10^{-2}$.

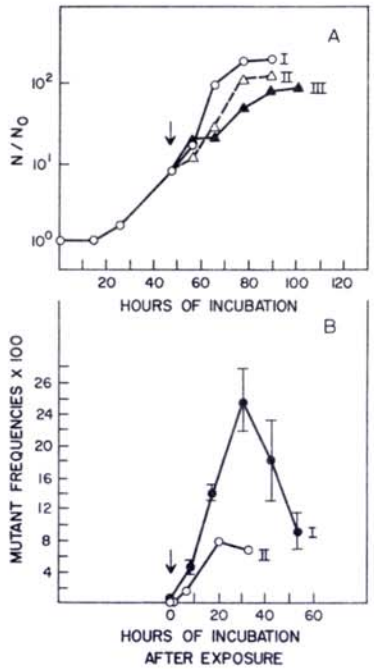

Fig. 3. A, Growth curves after UV irradiation of log-phase cultures with post-irradiation incubation on 0.025% caffeine. Cells were grown on Millipore with liver infusion medium. One set of cultures was left untreated to serve as control (○), curve *I*. At 46 hours the 5×10^4 cells inoculated per culture had increased about 10-fold. At this time (arrow) one set of cultures was transferred to caffeine medium and irradiated with 6 seconds of UV at dose-rate 8.5 ergs mm^{-2} sec^{-1} (▲), curve *III*. In the same experiment a third set of cultures were irradiated with the same dose of UV in absence of caffeine (△), curve *II* (see Fig. 2). One set of cultures was irradiated at the time of plating in presence of caffeine to be able to estimate induction of mutants under lag-phase conditions (growth curve not shown). B, Kinetics of appearance of mutants after UV irradiation of log-phase and lag-phase cells in presence of caffeine. Six seconds of UV at dose rate 8.5 ergs mm^{-2} sec^{-1} was applied to log-phase cells (●), curve *I*, and lag-phase cells (○), curve *II*, both in presence of caffeine. At time intervals indicated cells were harvested and plated for screening of plaque-size mutants. Viability was variable from about 5% to 75%. Abscissa: hours of incubation. Ordinate: mutant frequencies $\times 100$

(b) Mutants in Caffeine Grown Clones. The cells plated on caffeine were grown for 70–80 hours and inspected under the dissecting microscope. In the 98 clones examined, the average number of cells was 47. Thus 5–6 division cycles occurred during growth on caffeine. These clones were removed quantitatively from the plate and replated on water agar. After 7 days at 25°C each clone could be analysed for its plating efficiency and presence of plaque-size mutants. The 98 clones analyzed were arbitrarily divided into two classes according to the range of plating efficiencies encountered. The first class, consisting of 53 clones, had high plating efficiencies, in the range from 25% to 100% with an average of 44.5%. 37 of these clones contained one or more plaque-size mutants. The frequency of mutants in the class as a whole was $(10.0 \pm 0.9) \times 10^{-2}$.

The other class, consisting of 45 clones, had low plating efficiencies, in the range of 0% to 24%, with an average of 9.3%. Twelve of these clones contained one or more mutants at a total frequency of $(9.7 \pm 1.9) \times 10^{-2}$.

We conclude that the observed frequency of plaque-size mutants is not related to plating efficiency. More important, these results give indisputable evidence that caffeine-induced mutagenesis has occurred. In the original population only one mutant occurred among 932 cells. Thus the probability that any of the 98 clones analyzed contained any mutants at the time of plating on caffeine is very small. After 5–6 divisions cycles on caffeine half of the clones contained one or more mutants.

(c) Mutants in Clones Exposed to UV and Caffeine. Cells were plated on 20 ml of liver infusion agar and incubated for 70 hours at 25°C and the resulting clones (containing about 150 cells) were transferred to 20 ml plates with 0.05% caffeine. 4 hours was allowed for equilibration to 0.025% caffeine concentration, and the plates were then exposed to 50 erg mm^{-2} of UV and incubated for 24 ± 2 hours. At this point 97 clones were analysed and found to contain an average of approximately 300 cells, indicating one cell doubling during the 24 hours of post-irradiation incubation. Each clone was analyzed by quantitative replating on water agar, as described above.

After 7 days of incubation, plating efficiency and mutant frequency was determined. Plating efficiency was expected to be low because of the lethal synergism between UV and caffeine. The 97 clones were again divided into two classes according to the range of plating efficiencies. The first class, consisting of 39 clones, had plating efficiencies ranging from 5% to 33% with an average of 10.5%. Thirty-six of these clones contained one or more plaque-size mutants at a total frequency $(16.6 \pm 1.3) \times 10^{-2}$. The other class, consisting of 58 clones, had plating efficiencies in the range from 0% to 4%, with an average of 2.5%. 45 of these clones gave one or more mutants at a total frequency of $(22.9 \pm 2.2) \times 10^{-2}$.

We conclude that mutagenesis rather than selection must have occurred, since 81 out of 97 clones gave rise to one or more plaque-size mutants in the course of only 24 hours. In contrast only about 0.3% mutants would have been found without mutagenic treatment.

6. Induction of Cycloheximide Resistant Mutants

Genetic analysis of the plaque-size mutants was not feasible. In order to obtain evidence that nuclear mutants are induced by the methods described here, cycloheximide resistance was chosen as a marker, since at least one such mutant has been shown to be Mendelian (Dee, 1966b), and since such mutants could be analyzed genetically.

(a) Spontaneous Frequency of Cycloheximide Resistance. The spontaneous frequency of cycloheximide resistance was determined by plating a total of 4.3×10^8 untreated cells, from 78 separate cultures, on selection plates. Initially each of the cultures was inoculated with 5×10^4 cells. These were then grown to

10^6–10^7 before selection-plating. Only 4 mutants were obtained, each in a separate culture, indicating a frequency of $(9.3 \pm 4.7) \times 10^{-9}$.

(b) Induction of Cycloheximide Resistant Mutants by Growth on Caffeine. The frequency of cycloheximide resistant mutants among cells grown for 4 days (until late log phase, starting with an inoculum of 10^5 cells) on caffeine gave this result; all together 9×10^7 caffeine-grown cells from 17 different cultures were inspected; 6 of these culture gave rise to one mutant each. It is concluded that these mutants are independent and occur at a frequency of $(6.7 \pm 2.7) \times 10^{-8}$. Thus caffeine treatment alone marginally increases cycloheximide-resistant mutants and greatly increases plaque-size mutants over the spontaneous level.

(c) Induction of Cycloheximide Resistant Mutants by UV in the Presence of Caffeine. For this study the treatment which gave optimal conditions for induction of plaque-size mutants was used. Cultures were grown on Millipore membranes from inocula of 5×10^4 cells and transferred to caffeine medium in log-phase. Each such culture was given about 50 ergs mm^{-2} of UV and incubated another 24 hours. Cells were harvested and plated on selection plates.

At the same time, the titer was determined to estimate survival and frequency of plaque-size mutants. A total of 7.3×10^7 viable, mutagenized cells from 74 different cultures was screened. Eight cultures gave rise to one mutant each, while one culture gave rise to 6 mutants. The latter 6 showed similar characteristics and were assumed to have a single origin (probably spontaneous). Thus we conclude that 9 independent mutants were found at a frequency of $(1.2 \pm 0.4) \times 10^{-7}$. Caffeine plus UV, then, increases the frequency of cycloheximide resistant mutants by 13-fold and plaque-size mutants by 125- to 250-fold above the spontaneous level.

B. Mutant Selection by 5-Bromodeoxyuridine Sensitization

1. Conditions for Light Sensitization of Myxamoebae by BUdR

Bacteria had to be used as food for the myxamoeba. Thus, BUdR substitution of amoebae was achieved by feeding them with bacteria whose DNA had been substituted with BUdR (see Methods).

In an attempt to inhibit *de novo* thymidylate synthesis, and increase their BUdR content, myxamoebae were grown with BUdR-substituted bacteria on plates containing FUdR, an inhibitor of thymidylate synthetase (Bosch, Harbers and Heidleberger, 1958) plus uracil (50 μg per ml). FUdR at 10 and 30 μg per ml did not greatly increase the sensitization, so we assume it was not very effective in increasing the BUdR substitution of amoebae. Furthermore, growth was slightly inhibited at the highest concentration, and it was decided to omit FUdR from the medium.

Since incubation at 30°C selects against the heat sensitive mutants we hoped to isolate, preliminary experiments were performed to determine the minimum time required to incorporate enough BUdR to permit significant light sensitization. Light-inactivation curves obtained after various periods of BUdR incorporation showed that mutant enrichment was 10-fold after 10 hours of growth, 160-fold after 22 hours and 300-fold after 33 hours. Enrichment above 300- to 500-fold has rarely been achieved, since growth occurring at 30°C beyond 30–35 hours tends to obscure any enrichment by irradiation. As a result of these experiments, we have usually allowed 30 hours for BUdR incorporation at 30°C.

2. Results of Limited Selection

Log-phase cells were mutagenized with EMS and NMG as described in Methods, incubated with BUdR-bacteria at 30°C for 30 hours, and then light-inactivated. The survivors were plated singly and plaques allowed to develop at 23°C. The resulting clones were then screened for heat-sensitivity. Total enrichment in the EMS-mutagenized population was 100-fold, and in the NMG-mutagenized population 50-fold. 293 clones of such EMS-mutagenized cells were screened and one heat-sensitive mutant was found. This mutant, called *ts* E313, occurs at a frequency near 3×10^{-3} after selection, or 3×10^{-5} after mutagenesis alone.

400 clones from NMG-mutagenized, selected cells were screened and 2 heat sensitive mutants were found. These mutants, called *ts* N311 and *ts* N312, have different characteristics, and occurred at frequency near 5×10^{-3} after selection, or 1×10^{-4} after mutagenesis.

To show that the three heat-sensitive mutants obtained were indeed selected in the light sensitization procedure, the following reconstruction experiment was performed. Strain RSD4 *act B* N211 (Haugli, Jimenez and Dove, 1972) was mixedly plated with 1% of each of the three mutants and with 1% of wild type RSD4 (for control) and incubated with BUdR-bacteria at 30°C for 26 hours. Single cells were plated after light-inactivation and allowed to form plaques at 23°C. The resulting clones were then screened for cycloheximide resistance. The proportion of sensitive cells increased from 1% to 70–90% in the three cases involving mutants, while the proportion of sensitive cells in the control was not significantly altered. Thus, enrichment for the cycloheximide sensitive, heat sensitive cells has indeed occurred. Preliminary characterization of the three heat sensitive mutants included growth curves at 30°C and 25°C (the mutants grow better at 25°C than at 23°C). These are shown in Fig. 4. It is seen that all mutants are leaky, but to different degrees.

When cells of the three mutants were plated, 100 at a time, and incubated for various times at 30°C before transfer to 23°C, it was found that the plating efficiency remained high for the period tested (100 hours at 30°C). Thus, incubation at 30°C had no lethal effect.

Preliminary genetic crosses show that all three mutants segregate as single Mendelian factors.

3. Results of Extensive Selection

(a) Light Inactivation in Populations Exposed to Repated Cycles of BUdR Sensitization. To obtain mutants after a single cycle of selection it was necessary to screen a large number of clones. In an attempt to avoid this tedious procedure, we cycled the survivors of light inactivation to allow further BUdR incorporation, followed by a second round of light inactivation and recovery at 23°C. If the selection per cycle is about 300-fold, multiple cycles should result in an overall selection factor of 300 raised to the power of the number of cycles employed. It can be seen in Fig. 5 that each successive round of selection has decreasing

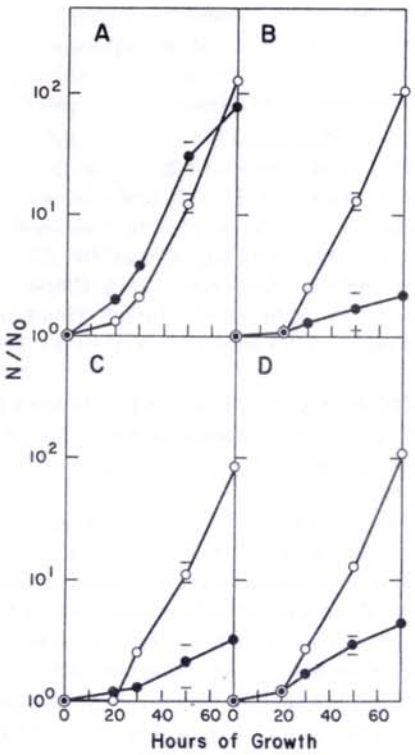

Fig. 4A—D. Growth curves of wild type (RSD4) and three heat sensitive mutants derived from it. Growth at 25°C (○); growth at 30°C (○). A, wild type, RSD4. B, mutant *ts* N311. C, mutant *ts* N312. D, mutant *ts* E313. All strains were inoculated 10^5 at a time with regular bacteria. Duplicate plates were harvested at times indicated, and cells counted in a hemocytometer to determine yield. 80% confidence limits were calculated as described in Methods. Abscissa: hours of incubation. Ordinate: N/N_0, on a logarithmic scale where N is cell yield at any one time and N_0 the inoculum

selection power. Surprisingly, the majority of cells remaining after multiple cycles can grow at 30°C; this procedure appears to select for BUdR resistant cells, rather than for heat sensitive mutants of DNA synthesis.

(b) Screening for Heat Sensitive Mutants in Extensively Selected Populations. Cells which had been taken through 4 selection cycles were screened for heat sensitive mutants. Among 500 clones, no such mutants were found.

(c) Clonal Analysis of Extensively Selected Populations. What type of cells are present in a population after 4 cycles of selection? Light sensitization of wild type, an extensively selected population and 15 clones isolated from such selected populations were studied. These various cell lines were grown at 25°C on BUdR-bacteria for 5–6 division cycles. The wild type cells could be light-sensitized but the selected population and its isolated clones could not (one clone, N56, exhibited partial sensitivity). These cell lines, therefore, represent a novel class of

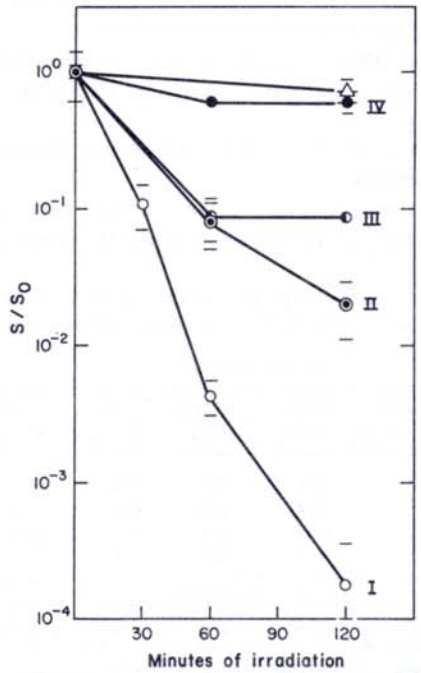

Fig. 5. Light sensitization of mutagenized myxamoebae in successive cycles of light inactivation after growth at 30°C on BUdR substituted bacteria. Curve *I* (○): cells were mutagenized with NMG, grown 30 hours at 30°C (3 division cycles) with BUdR substituted bacteria, and light inactivated as described in Methods. Curve *II* (⊙): surviving cells (from end point of curve *I*) recovered at 23°C were resensitized with BUdR (24 hours at 30°C; 2 division cycles). Curve *III* (◐): surviving cells were resensitized with BUdR, as above (30 hours at 30°C; 2 division cycles). Curve *IV* (●): in this final selection cycle survivors of cycle III recovered at 23°C and were then incubated for 35 hours at 30°C with BUdR bacteria (4 division cycles). Irradiation gave the survival curve shown. Curve with symbol (△) shows the survival of irradiated, unsubstituted cells. All data obtained by duplicate titering. 80% confidence limits determined as described in Methods. No limits shown means that the range is smaller than the symbol. Note: original population of 2×10^7 viable, mutagenized cells was divided in 4 batches at each light inactivation step. The light inactivation curves shown are therefore representative samples of these subgroups. Certain subgroups were lost due to contaminations, and the final number of such cell populations taken successfully through 4 cycles was 7. These all appeared to be populations fully resistant to BUdR sensitization. Abscissa: minutes of irradiation. Ordinate: S/S_0 on a logarithmic scale, where S is viable titer at any one dose and S_0 the initial viable titer

mutants. We shall call the wild type allele bu^+, and the mutants bu^r. Since all of these mutations have been obtained in RSD4 after NMG mutagenesis, they will be named in agreement with our earlier nomenclature (Demerec, Adelberg, Clark and Hartman, 1966; Haugli, Jimenez, and Dove, 1972), as RSD4 bu^rN, followed by a strain number.

(d) Biochemical Characterization of a BUdR Resistant Mutant. One can imagine several types of mutants resisting light-sensitization by growth on BUdR substituted bacteria:

1. Mutants unable to utilize BUdR from bacterial DNA. Among these, three subclasses can be distinguished:

1a. Those lacking a DNAase which degrades bacterial DNA;

1b. Those lacking enzymes(s), for example a kinase, necessary for operation of the salvage pathway;

1c. Those mutants in which the DNA polymerase selects against BUdR and only incorporates thymidine.

2. Mutants containing a special system which can repair the (normally irreparable) lesions caused by illumination of BUdR-substituted DNA.

Table 1. Determination of radioactivity (μCi per cell) in DNA, RNA and protein (P) after growth to encystment of myxamoebae on labeled bacteria

Strain	Expt. no.	μCi per cell × 10^9					
		Label: TdR	BUdR	TdR	BUdR	TdR	BudR
		Fraction: DNA	DNA	RNA	RNA	P	P
WT	1	9.4	4.8	0.8	16.0	3.8	1.5
MUT	1	0.4	1.2	0.6	13.0	1.8	0.9
WT	11	12.8	10.3	2.8	25.3	12.4	7.8
MUT	111	0.5	3.6	1.4	27.6	2.6	2.2
Ratio	WT 1/MUT 1	23.5	4.0	1.3	1.2	2.1	1.7
Ratio	WT 11/MUT 11	25.6	2.9	2.0	0.9	4.8	3.5

Strains: RSD4 (wild type = WT) and RSD4 *bu*^{r} N52 (mutant = MUT). TdR labeled bacteria had specific activity 1.5×10^{-9} μCi/bacterium and BUdR labeled bacteria had specific activity 4.1×10^{-9} μCi/bacterium. The lower activities in experiment no. 1 is due to the dilution (1:1) of labeled with unlabeled bacteria.

These possibilities were tested by feeding wild type and mutant amoebae with labeled bacteria containing either methyl-tritiated TdR or ring-tritiated BUdR. DNA, RNA and protein were then extracted. Wild type RSD4 and mutant *bu*N^{r}52 were selected for this work because these strains are isogenic except for the mutation conferring resistance to BUdR light-inactivation. *E. coli* CR34 was grown in minimal medium containing either 5-bromo-2-deoxyuridine-6-H^3 at specific activity 1.25 μCi per μg (Schwarz, cat. no. 2733-87, lot no. 6602, radiochemical purity 98%), or thymidine-methyl-H^3, at specific activity 1.25 μCi per μg (New England Nuclear, cat. no. NET-027, lot no. 560-087). The radioactive bacteria were formalin-inactivated, washed and fed to the wild type and mutant cells (for details, see Haugli, 1971). Incubation was continued until depletion of the bacteria and encystment of the amoebae. Encysted amoebae were further purified from residual bacteria by centrifugation through a 40 ml linear gradient from 0.5 M to 1.5 M sucrose in 0.01 M $CaCl_2$, 0.01 M Tris pH 7.0 (a gradient developed by Dr. Justin McCormick, McArdle Laboratory, for purification of nuclei). After 50 minutes at 500 rpm in Sorvall rotor HB 990, the broad band of cells near the bottom of the tube was completely separated from remaining bacteria and debris. The purified cells were washed in water, and counted in a hemocytometer. RNA, DNA and protein were extracted, and radioactivity

measured by scintillation counting in Scintisol. Two experiments gave similar results (Table 1). It can be seen that the amount of label incorporated into RNA and protein is similar in wild type and mutant. Thus, TdR and BUdR are degraded and reutilized in both systems. These results exclude the possibility (1a above) that the mutant, *bu*^{r}N52, cannot degrade bacterial DNA.

It was found that the mutant is only slightly deficient (3–4 fold) in utilization of the 6-H^3 provided by BUdR.

In contrast, the mutant is 25-fold deficient in utilization of the methyl group provided by TdR. By implication, we suppose that the mutant would also be 25-fold deficient in utilization of intact BUdR.

Metabolic conversion of TdR and BUdR followed by reuse of salvaged fragments in *de novo* thymidylate synthesis appears to nearly cancel the differences between wild type and mutant when the label is provided in the ring. However, the great difference observed between mutant and wild type when the label is provided in the methyl group leads us to conclude that mutant *bu*^{r}N52 is deficient in some metabolic step necessary for reutilization of this group in a salvage pathway, as in possibility 1b above. Alternatively, the mutant could have an increased demethylase activity.

Discussion

A. UV and Caffeine-Induced Mutagenesis

1. Effect of Growth on Appearance of UV-Initiated Mutants

The importance of the post-irradiation treatment for the appearance of UV-initiated mutants has long been known (see, for example, Witkin, 1958). Irradiation of log-phase cells with low doses of UV, followed by various periods of incubation, showed that mutants appeared as a function of the time of incubation (Fig. 2). Further, it was found that growth occurring between irradiation and plating, rather than time itself, is a critical factor in mutagenesis (Fig. 2). Thus if the premutational lesions induced by UV are to appear as mutations, active growth is needed. This could refect a need for fixation and/or segregation. The experiment with lag-phase cells (Fig. 2) suggests that at least fixation is involved. Twenty hours after irradiation, when the mutant yield in the log-phase cultures had increased about 13-fold, the lag-phase cells had not yet started to increase their numbers, and the mutant yield was only slightly above background. At 32 hours, however, these lag-phase cultures had nearly doubled, and still no increase in mutant frequency was observed. This result indicates that the potential which was present at the time of irradiation was lost during the lag-phase, suggesting the presence of repair mechanisms.

2. Effects of Caffeine on UV-Induced Inactivation and Mutagenesis

The extensive shoulder of the UV inactivation curves for *Physarum* myxamoebae could reflect either multi-hit targets, or a repair activity which reduces the effective UV dose until the number of lesions has saturated the repair system. If we interpret the shoulder as arising from multihit targets, the multiplicity is in the range of 1000, as judged by extrapolation of the "final" slope back to the ordinate (Fig. 1). It is striking that caffeine abolishes the shoulder on the UV

inactivation curve. Since it is difficult to explain this effect in terms of decreasing the target number from about 1000 to about 3, we suppose that caffeine directly affects a repair system.

It has been shown that mutants of *E. coli* lacking excisional repair have lost caffeine sensitization for both UV-induced killing (Sauerbier, 1964; Metzger, 1964) and mutagenesis (Clarke, 1967; Sideropoulos and Shankel, 1968). This suggests that the effects of caffeine in *E. coli* are expressed through a mechanism which is connected to excisional repair. By analogy, then, caffeine-induced UV sensitization in *Physarum* may occur through inhibition of excisional repair. Of course, direct evidence is required to prove this suggestion.

Log-phase cells exposed to 50 ergs mm^{-2} of UV showed little decrease in viability and only a moderate, growth dependent increase in frequency of plaque-size mutants. The same dose of UV given to similar cultures in the presence of caffeine produced a large decrease in viability and a growth dependent, 100- to 200-fold increase in mutant frequency among survivors (Fig. 3). Clonal analysis showed that this effect was primarily a result of mutagenesis, rather than selection.

It was also shown that the frequency of cycloheximide resistant cells was stimulated 13-fold under optimal conditions. Here the evidence against selection was provided by a limited fluctuation test (Luria and Delbrück, 1943).

3. Effects of Caffeine Alone on Mutant Yield

When myxamoebae were incubated on caffeine-containing medium for extended periods (70–80 hours, corresponding to 5–6 division cycles) the frequency of plaque-size mutants increased 60-fold or more. That this increase is not caused primarily by selection was shown by clonal analysis. The maximum mutagenic effect of caffeine was during log-phase of growth. Similar caffeine treatment resulted in a 7-fold enhancement in the frequency of cycloheximide resistant cells. Here the evidence for mutagenesis was also provided by a limited fluctuation test.

While the mode of action of caffeine in enhancing or diminishing the mutagenic effects of UV is poorly understood, even less is known about the effects of caffeine alone and the data in the literature are conflicting (Fries, 1950; Novick and Szilard, 1951; Witkin, 1958; Grigg and Stuckey, 1966; Kao and Puck, 1969; Trosko and Chu, 1971). In the absence of more extensive data, or a real understanding of how caffeine acts, it is not possible to propose any mechanism for the strong, growth dependent mutagenic action of caffeine on *Physarum* myxamoebae.

B. Mutant Selection by 5-BUdR

The present communication shows that indirect selection for heat sensitive mutants can be applied to *Physarum polycephalum*. The method, used successfully with bacteria and mammalian cells in culture, is designed to give mutants with heat sensitive DNA synthesis. Its application in *Physarum* is complicated by the occurrence of a class of mutants defective in the direct utilization of exogenous thymidine or BUdR at any temperature. A further study of these mutants will be undertaken.

Extensive selection could be applied without outgrowth of mutants defective in thymine (and BUdR) utilization, if a thymine auxotroph were available. Such auxotrophs could not grow if they could not directly utilize exogenous thymidine (and BUdR).

Three heat sensitive mutants were selected and, in preliminary experiments, the mutant characters were shown to segregate as single Mendelian factors. These mutants are now being studied further, genetically and biochemically.

Continued selection for heat sensitive mutants by this method may provide a complete genetic description of the functions necessary for DNA synthesis in *Physarum polycephalum*. The natural synchrony of the nuclear cycle in this eukaryote might be expected to facilitate this approach to the study of DNA synthesis. Biochemical studies of the nuclear cycle of *Physarum* have been summarized by Braun and Wili (1969) and by Cummins (1969).

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