Arch. Mikrobiol. 70, 313–329 (1970)

Action of Mutagenic Chemicals on *Anacystis nidulans*

V. Diethyl Sulphate

R. S. Gupta and H. D. Kumar

Department of Botany, University of Udaipur, Udaipur, India

Received July 28, 1969

Summary. A mutant strain of the blue-green alga *Anacystis nidulans* showing a 1000-fold increased resistance to streptomycin over the parental strain was isolated following treatment with diethyl sulphate. The mutant strain was found to show profuse filamentation, partial requirement of streptomycin for pigment production, and greater resistance to ultraviolet radiation. It also showed greater sensitivity to dimethyl sulphate than the parental strain. It seems that the streptomycin-resistant strain is a composite one comprising at least four kinds of mutations, namely streptomycin-resistance, filamentation, u.v.-resistance and partial dependence on streptomycin for pigment accumulation. Further, the streptomycin-resistant mutant seems nuclear rather than extra-nuclear in nature.

Treatment with diethyl sulphate also conferred a slight increase in resistance to penicillin in this alga.

During the past two decades various chemical mutagens have been employed as effective analytical tools for the study of nuclear material and genetic phenomena in diverse kinds of organisms. The alkylating agents, which constitute a class of potent mutagens, have been shown to induce gene mutations, deletions and other types of chromosomal rearrangements in various plants (Auerbach, 1967). Very little work has, however, been done on the action of alkylating agents on the blue-green algae (*Cyanophyceae*), a group of photosynthetic, procaryotic organisms characterized by the lack of conventional type of chromosomes, and whose genetic processes seem more closely akin to those of bacteria than to those of other classes of algae (Kumar, 1962; Singh *et al.*, 1966). The only previous study of the action of an alkylating agent on blue-green algae was that of Kumar (1968) who reported that ethyl methane sulphonate effectively increased the proportion of streptomycin-resistant mutants in treated populations of *Anacystis nidulans*.

In all organisms beyond the evolutionary level of the Procaryota, the chromosomes are complex structures containing proteins and RNA in addition to DNA, and it would be logical to assume that the role of such a chromosomal complexity in the production of mutations is likely to be important. The circumstance that virtually nothing is known

about this aspect of mutagenesis and that most of the previous studies on the action of alkylating agents have been carried out either on higher plants or on bacteria, made it worth while to study the problem in a blue-green alga. The alkylating agent diethyl sulphate was specially chosen for this work because it has been known to preponderantly induce gene mutations, to the almost total exclusion of chromosomal rearrangements (Auerbach, 1967).

A preliminary study of the effect of diethyl sulphate on *Anacystis nidulans* was undertaken by Kumar (1965) and Shestakov and Jevener (1968) have recently described the mutagenic action of diethyl sulphate on the same organism.

Material and Methods

The axenic and clonal strain of *Anacystis nidulans* used in this study was the same as in previous work (Kumar, 1964). Its suitability as an experimental organism for genetical and physiological studies has also been described (Kumar, 1964).

The alga was grown in a slightly modified Medium C of Kratz and Myers (1955), of the following composition in grams per litre of pyrex distilled water: K_2HPO_4, 1.00; KNO_3, 1.00; $Ca(NO_3)_2 \cdot 4\,H_2O$, 0.025; $MgSO_4 \cdot 7\,H_2O$, 0.25; Na_3-citrate $\cdot\, 2\,H_2O$, 0.20; $FeCl_3 \cdot 6\,H_2O$, 0.004; and 1.0 ml of an A_5 micronutrients stock solution containing in grams per litre of pyrex glass distilled water, H_3BO_3, 2.75; $MnCl_2 \cdot 4\,H_2O$, 1.80; $ZnSO_4 \cdot 7\,H_2O$, 0.20; MoO_3, 0.06; $CuSO_4 \cdot 5\,H_2O$, 0.05, and $Co(NO_3)_2 \cdot 4\,H_2O$, 0.05. The phosphate was autoclaved separately to avoid precipitation and added to the medium after cooling. The pH of the medium was adjusted with NaOH to approximately 8.0 after autoclaving and mixing of phosphate.

For growth on agar, the method suggested by Allen (1968) was followed. The medium employed was a modification of Hughes *et al.* (1958) medium in the sense that ferric citrate was replaced by ferric chloride and sodium molybdate by molybdenum trioxide. The actual composition of the medium used in grams per litre of pyrex distilled water was: $NaNO_3$, 1.5; $MgSO_4 \cdot 7\,H_2O$, 0.075; $Na_2SiO_3 \cdot 9\,H_2O$, 0.058; K_2HPO_4, 0.039; $CaCl_2$, 0.027; Na_2CO_3, 0.02; Na_3-citrate $\cdot\, 2\,H_2O$, 0.006; $FeCl_3 \cdot 6\,H_2O$, 0.006; EDTA, 0.001, and 1.0 ml of the A_5 trace element stock solution as described above. The agarized medium was prepared by the addition of 3% agar in distilled water, which after autoclaving separately was added to the double strength culture medium; upon mixing of the two, a final concentration of 1.5% agar in normal strength medium was obtained. Each plate (8 cm diameter) contained 20 ml of medium. Cells were seeded with the help of a glass spreading rod and the inoculated plates incubated inverted in culture chambers.

Cultures were grown at $38 \pm 2°C$ in a cabinet illuminated by a 100-watt tungsten filament lamp from a distance of 30 cm. The light intensity at the level of culture flasks varied between 350 to 450 lux.

Growth was estimated by counting the number of cells in a Neubauer haemacytometer and also by measuring the optical density of the culture tubes in a Bausch and Lomb Spectronic-20 colorimeter set at a wavelength of 600 nm. Cell lengths were measured by means of a calibrated ocular micrometer and unicellular or multicellular filaments were treated as individual cells for this purpose. Growth in various cultures was determined mostly by the duration of the lag-phase, the final population and optical density during the postlogarithmic stationary phase, and by the specific growth rate k ($\log_{10} N/N_0 = kt$, where t is in days; Kratz and Myers, 1955).

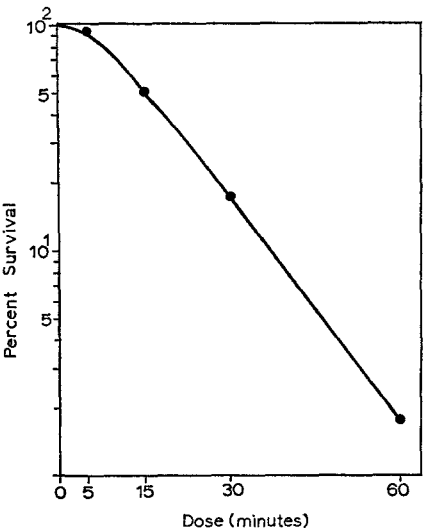

Fig. 1. Survival curve of diethyl sulphate treated *Anacystis nidulans*

To screen out the genetical variants resistant to streptomycin and penicillin from diethyl sulphate-treated populations, it was at first necessary to ascertain the range of sensitivity of the parent strain to streptomycin and penicillin. This was achieved by inoculating three-day old cells of *A. nidulans* in graded concentrations of the respective antibiotic. With these series of experiments, the maximum concentration of the drug permitting growth was determined. The maximum concentration of streptomycin permitting growth of untreated *A. nidulans* was found to be 0.05 μg/ml after a lag-phase of about 10—12 days, and the maximum concentration of penicillin permitting growth was 0.09 μg/ml after a lag of 9—11 days.

The diethyl sulphate dose-response curve was obtained by treating algal suspensions of same cell density with 0.1 M diethyl sulphate for 0, 5, 15, 30 and 60 min. After washing, the treated samples were plated on basal medium and both experimental and control plates were incubated in the culture cabinet. After two weeks of incubation, percentage survival was scored by colony counting. Fig. 1 indicates that a dose of 60 min is lethal and that DES killing of *A. nidulans* is exponential. Since the isolation and scoring of viable mutants require a dose of mutagen that permits a good number of treated cells to survive, a 15-min dose of diethyl sulphate giving approximately 50% survival was selected for isolation of diethyl sulphate-induced mutants resistant to streptomycin or penicillin.

Three-day old cells of *A. nidulans* were first concentrated by centrifugation to get about 3 ml inoculum suspension containing about $4.5—5.3 \times 10^6$ cells/ml. Two 150-ml conical flasks were sterilized with 9 ml of citrate-phosphate buffer (pH 5.0) in each. Upon cooling, 0.1 M diethyl sulphate was added into one flask while the other flask served as control. Into each flask was added 1 ml of the centrifuged algal suspension and the flasks shaken continuously for 15 min. The treatment was terminated by diluting the reaction mixture into 10 volumes of cold sterile Tris (0.05 M, pH 8.0), followed by two washings in sterile water. 0.2 ml samples from the control and treated flasks were then inoculated into culture tubes (150×25 mm)

containing 20 ml of medium and supplemented with appropriate concentrations of streptomycin or penicillin.

Pigment Extraction. Aliquots from control and treated flasks containing equal cell volumes were centrifuged at 4500 rpm for 30 min. The supernatant was discarded and the pellet washed once with distilled water, centrifuged and then cells suspended in 7—8 ml of 80% aqueous acetone. After stirring the tubes were kept in a refrigerator for 2 hr and then the contents centrifuged. The supernatant was made up to 10 ml by the addition of 80% acetone in a volumetric flask. The absorption spectrum of each extract so obtained was recorded in the wavelength range of 400—700 nm in test tubes of 1.2 cm diameter.

Ultraviolet Irradiation. 15 ml volumes of log-phase cell suspensions of known cell density in sterile water and contained in 4.5 cm petri dishes were exposed to graded doses of u.v. light at a distance of 18 cm from a Philips germicidal lamp (main emittance at 2537 Å, 96 ergs/mm^2/sec). The suspension was constantly stirred on a magnetic stirrer during irradiation. The lids of petri dishes were removed during irradiation.

Results

Production and Isolation of Resistant Strain: Three-day old cells were treated with 0.1 M diethyl sulphate for 15 min in acid buffer (pH 5.0) and after washing inoculated into culture tubes containing basal medium. Samples of 22×10^5 cells each from 7-day old diethyl sulphate treated culture and from untreated culture were separately harvested and inoculated into basal culture tubes supplemented with nil, 0.0005, 0.005, 0.05, 0.25 and 0.50 µg streptomycin/ml. In these concentrations, treated cells grew after a lag of 1, 3, 9, 10, 13 and 28 days respectively, while the untreated cells grew after lags of 1, 3, 15 and 16 days in the first four concentrations; no growth of untreated cells occurred in higher concentrations. The specific growth rates k given in Table 1 suggest that diethyl sulphate treated cells can grow in higher concentrations of streptomycin than the parental strain.

To find out whether or not the increased resistance of diethyl sulphate treated population to streptomycin is mutational in nature, and if mutational, whether spontaneous or induced, a modified Luria and Delbrück (1943) fluctuation test was performed. The entire population of a 10-day old culture was harvested and the cells divided into two sets; a sample of 2×10^4 cells from one set was directly inoculated into each of the 12 freshly prepared basal culture tubes; the cells of the other set were further divided into 12 samples of 7×10^6 cells each and then each sample was given diethyl sulphate treatment of 15 min. Inocula containing 2×10^4 cells from each of the treated populations were inoculated into separate culture tubes containing basal medium. Both experimental tubes and controls were incubated under similar environmental conditions. At the end of the growth period, a sample (7×10^5 cells) each from the experimental and control series was taken and inoculated into fresh culture tubes containing 0.5 µg streptomycin/ml. Samples

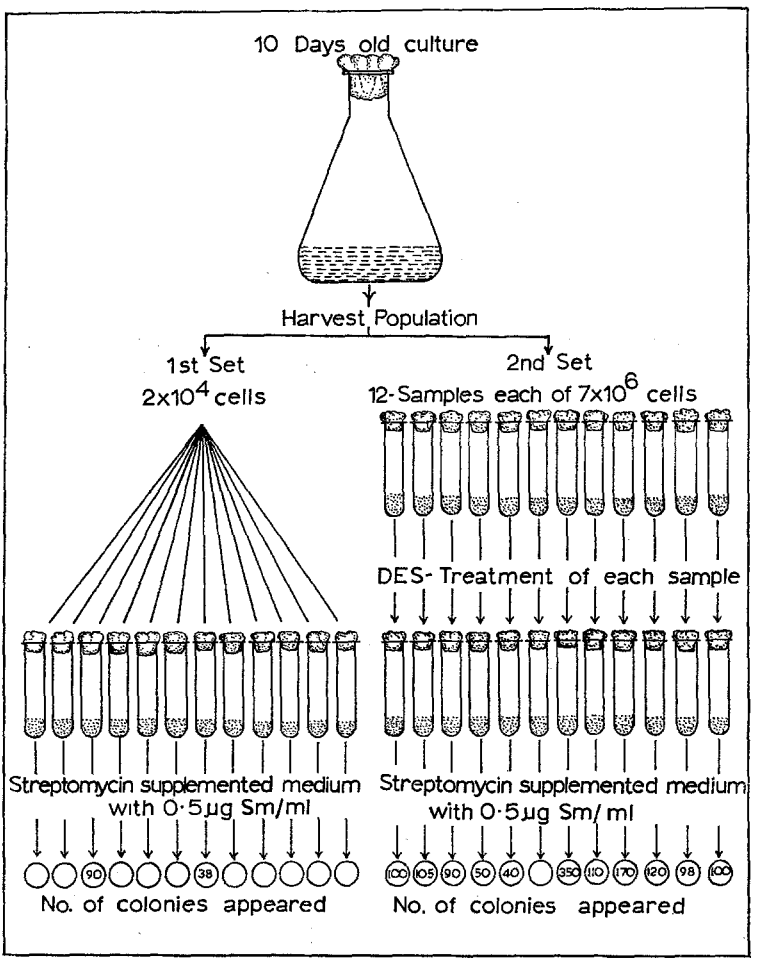

Fig. 2. Schematic representation of fluctuation analysis of diethyl sulphate induced streptomycin-resistant strain of *A. nidulans*

from 11 of the 12 tubes of experimental series grew in streptomycin media, whereas in the case of the controls, samples from only two tubes grew in streptomycin.

The liquid culture experiment of Luria and Delbrück fluctuation test was simultaneously conducted on agar medium also, using the same sized-inoculum as in liquid culture experiments. In streptomycin plates inoculated with progeny of diethyl sulphate treated suspensions, growing colonies were obtained from 11 of the 12 culture tubes. But in the case of control series, samples from only two cultures produced colonies on

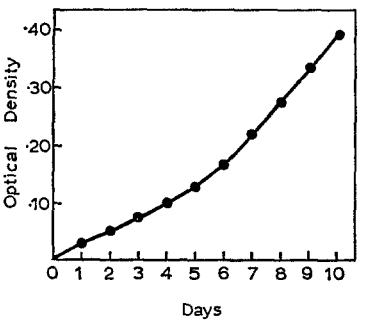

Fig. 3. Growth of diethyl sulphate induced streptomycin-resistant mutant in 50 μg streptomycin/ml after five successive subcultures in basal medium

streptomycin agar. The scheme followed in the fluctuation analysis is shown in Fig. 2. According to the fluctuation hypothesis of Luria and Delbrück (1943), if mutation to resistance is spontaneous then there should be a large fluctuation in the number of samples having streptomycin-resistant individuals, but if the mutation is induced in nature, then there should be little or no fluctuation. The experimental results described above suggest that the comparatively low fluctuation observed within the number of treated samples harbouring streptomycin-resistant cells is simply because of the induction of mutation to streptomycin-resistance by diethyl sulphate.

Six colonies were picked up at random from one of the streptomycin plates that had been inoculated with diethyl sulphate treated cells and these colonies were individually inoculated into culture tubes containing 0.5 μg streptomycin/ml. Growth occurred in these tubes after a lag of 2—3 days. Equal inocula from one of these cultures were further inoculated into higher concentrations of streptomycin (1.0, 5.0, 10.0, 25.0 and 50.0 μg/ml). Growth occurred in all the concentrations after a lag of 2—4 days. These observations suggest that diethyl sulphate induced mutation to streptomycin-resistance originally selected at 0.5 μg/ml has subsequently attained the resistance level of 50 μg/ml.

Stability of Resistance: The cells growing in 0.5 μg streptomycin/ml were subcultured in streptomycin-free basal medium 5 times and then inoculated into 6 culture tubes each supplemented with 0.5 μg streptomycin/ml. Growth occurred in all the 6 tubes, showing thereby that the streptomycin-resistance is a stable character.

A further test for stability of resistance was performed by subculturing a 50 μg streptomycin/ml-resistant strain in basal medium 5 times and then re-inoculating the cells into media containing 50 μg strepto-

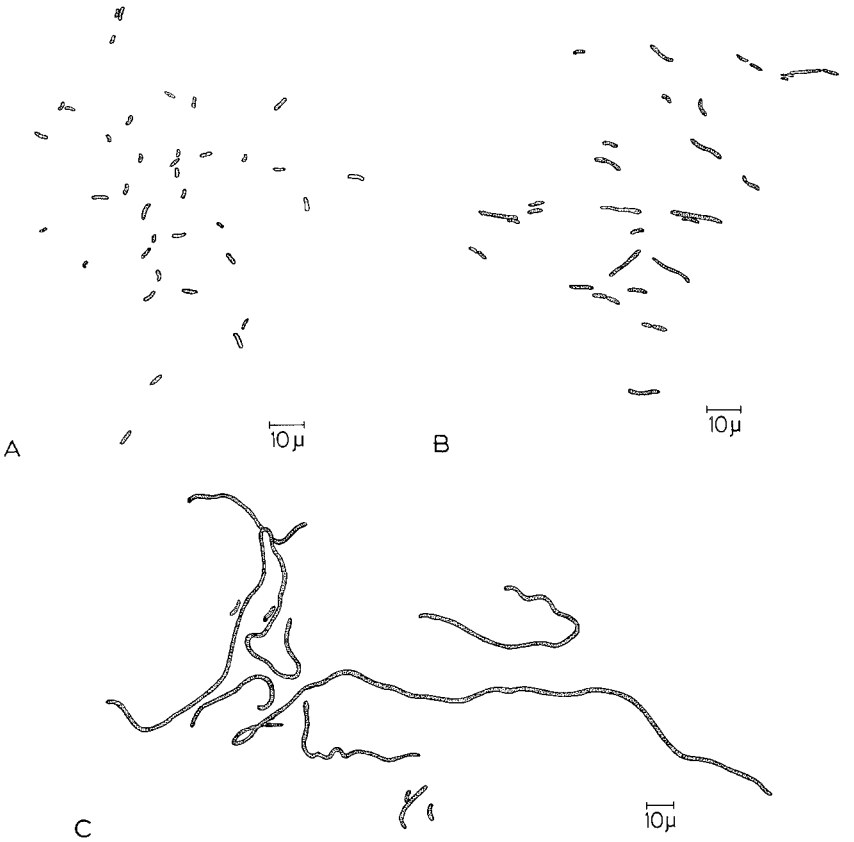

Fig. 4. A. Cells of parental (control) strain of *A. nidulans*. B. Cells of diethyl sulphate induced streptomycin-resistant mutant in 50 μg streptomycin/ml. C. Diethyl sulphate induced abnormally long, coiled filaments of *A. nidulans* in 0.5 μg streptomycin/ml

mycin/ml. Growth occurred in this concentration without a lag, indicating thereby the stable nature of streptomycin-resistance (Fig. 3).

Effect on Cell Length: Diethyl sulphate treated cells that had been inoculated in different concentrations of streptomycin (up to 0.5 μg/ml) were examined microscopically on the 15th day following inoculation and were found to exhibit abnormally long coiled filaments in addition to normal cells (Fig. 4C). These filaments were longer and more numerous in the lower concentrations of streptomycin than in the higher. The maximum length attained was 1000 to 2000 times greater than that of the normal parental cells. In all successive transfers in media containing less than 50 μg streptomycin/ml, the filamentous population persisted.

However, the cells growing in 50 μg streptomycin/ml were invariably longer than those in untreated controls (Figs. 4 A and B). The increase in cell length of the strain resistant to 50 μg streptomycin/ml, as compared to the parent strain, was found to be highly significant at $P = 0.01$ level ($t_{(40)} = 5.263$).

Characterization

1. Growth of Streptomycin-Resistant Strain in Higher Concentrations of Streptomycin

The diethyl sulphate induced 50 μg streptomycin/ml-resistant strain when inoculated (4×10^5 cells/ml) in duplicate tubes containing 50, 100, 200 and 500 μg streptomycin/ml of basal medium, never grew in any of the higher concentrations. However, when a series of 6 parallel samples, each of 2×10^5 cells, were withdrawn from two different cultures of 50 μg streptomycin/ml-resistant strain and inoculated into culture tubes containing 100 μg streptomycin/ml, growth did occur in one tube out of 12 after a lag of 20 days. A similar type of experiment was conducted from cells grown in 100 μg streptomycin/ml and inoculated in 500 μg/ml but no growth occurred in any tube in this experiment.

When inocula from the culture in 100 μg streptomycin/ml were subcultured in media supplemented with the same concentration of streptomycin, growth occurred without a lag. Further successive subculturings of this strain in basal medium did not result in any loss of its resistance level. It may therefore be concluded that the 100 μg/ml-resistant strain arose in one of the cultures containing 50 μg/ml-resistant cells, and was then selected by the screening concentration. Moreover, the isolation of a 500 μg/ml-resistant strain directly from a 50 μg/ml-resistant strain was never successful and the 500 μg strain could only be obtained from 100 μg strain by a method exactly similar to the one adopted for isolation of 100 μg/ml-resistant strain from the 50 μg/ml-resistant strain. This suggests that 500 μg streptomycin/ml-resistant strain might have arisen from 100 μg strain in the same way in which the 100 μg strain arose from 50 μg strain.

Table 1. *Specific growth rates* (k) *of diethyl sulphate treated and untreated cells of* Anacystis nidulans *in different concentrations of streptomycin*

Streptomycin, μg/ml	*k*	
	treated	untreated
0.0005	0.380	0.425
0.005	0.328	0.395
0.05	0.244	0.380
0.25	0.236	0.0
Nil (Control)	0.460	0.468

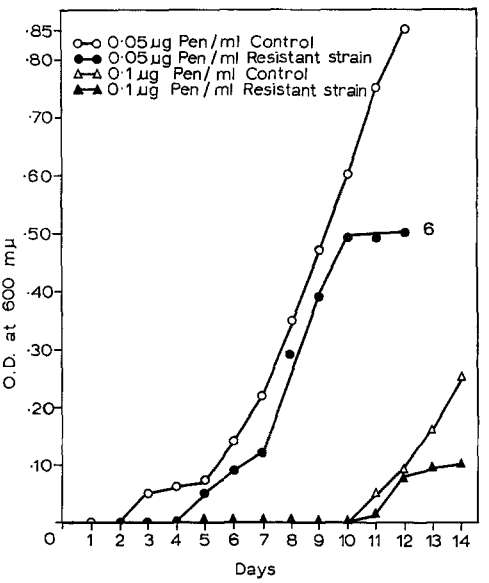

Fig. 5. Effect of penicillin on growth of diethyl sulphate induced streptomycin-resistant mutant of *A. nidulans*

2. Penicillin

A triplicate set of culture tubes containing different concentrations of penicillin (0.05, 0.1, 1.0 and 10.0 µg/ml) was inoculated with diethyl sulphate induced streptomycin-resistant cells or with untreated control cells. Growth of both resistant and parent cells occurred in tubes containing 0.05 and 0.1 µg penicillin/ml (Fig. 5). No significant differences in their relative tolerance to penicillin were found. Thus the diethyl sulphate induced streptomycin-resistant strain is not cross-resistant to penicillin.

3. Proflavine

The resistant strain was inoculated into triplicate tubes containing 0.1 and 1.0 µg proflavine/ml. Growth occurred in 0.1 µg/ml after a lag of 2 days but no growth occurred in the higher concentration even after 10 days. To test the stability of streptomycin-resistance after treatment with proflavine, cells of the resistant strain grown in 0.1 µg proflavine/ml were inoculated in the same concentration of streptomycin, i.e., 50 µg/ml. Growth occurred without a lag (Fig. 6) indicating thereby that streptomycin-resistance is not lost after treatment with proflavine.

The persistence of diethyl sulphate induced streptomycin-resistance even after proflavine treatment suggests that the induced resistance is nuclear rather than cytoplasmic in nature.

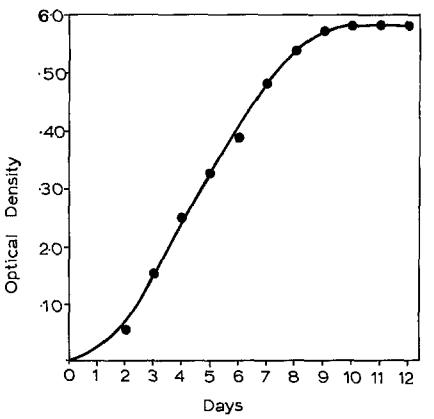

Fig. 6. Growth of induced streptomycin-resistant strain in 50 μg streptomycin/ml after treatment with 0.1 μg proflavine/ml

4. Maleic Hydrazide

Duplicate culture flasks containing 100, 500 and 750 μg maleic hydrazide/ml were inoculated with streptomycin-resistant cells and normal cells. No differences in their tolerance to maleic hydrazide were found and both the strains grew in up to 500 μg maleic hydrazide/ml, thus suggesting that streptomycin-resistant strain and parental strain are equally resistant to maleic hydrazide.

5. Dimethyl Sulphate

The streptomycin-resistant strain was inoculated into duplicate flasks supplemented with 0.01 ml (the maximum concentration permitting growth of parent strain) and 0.05 ml dimethyl sulphate/ml. The absence of growth in both the concentrations indicated that the diethyl sulphate induced streptomycin-resistant strain had become more sensitive to dimethyl sulphate than the parent strain.

6. Ultraviolet Radiation

Aliquots containing 9×10^7 cells/ml of resistant and normal cells (10-day old) were exposed to ultraviolet light for 2, 4, 6, 10, 15 and 30 min. After each exposure, 1 ml inocula were withdrawn and added into duplicate flasks containing 100 ml of basal medium. Growth was observed by noting the duration of the lag-phase and by measuring the final optical density on the 30th day in each case. In this way the two strains have been compared and characterized. The data obtained (Table 2) indicate that diethyl sulphate induced 50 μg streptomycin/ml-resistant strain shows a comparatively shorter lag and can grow after irradiation

Table 2. *Growth characteristics of induced streptomycin-resistant strain and untreated control strain of* A. nidulans *after irradiation with ultraviolet*

UV dose (min)	Resistant strain		Control (parent) strain	
	Lag (days)	O.D. on 30th day	Lag (days)	O.D. on 30th day
2	4	0.60	6	0.55
4	9	0.58	10	0.37
6	7	0.63	12	0.12
10	8	0.99	—	—
15	10	0.69	11	0.75
20	12	0.47	—	—
30	13	0.76	—	—

(Note: — = no growth).

with higher doses of ultraviolet than the parent strain. Thus, the streptomycin-resistant cells grew well after up to 30 min of ultraviolet exposure whereas the normal cells survived only a 15 min exposure. This indicates that the resistant strain can tolerate almost double the dose of ultraviolet tolerated by the untreated control cells.

7. Pigment Production

The 50 μg streptomycin/ml-resistant cells were inoculated in 50 μg streptomycin/ml and in basal medium. Cells of the parent strain were also inoculated in separate flasks containing basal medium. On the 10th day, acetone-soluble pigments were extracted from equal cell numbers (2×10^8 cells/ml) in all the three cases. The absorption spectra obtained indicate that the parental and diethyl sulphate induced streptomycin-resistant strains are similar in respect of the absorption peaks of chlorophyll-a (660 nm) and carotenoids (430 nm) but that the quantity of these pigments is lesser in the resistant strain than in the parental strain. The chlorophyll-a content in milligrams per 2×10^8 cells, as calculated by the formula of Whatley and Arnon (1963) is as follows: parental strain, 2.204; resistant strain in streptomycin medium, 1.334, and resistant strain in basal, streptomycin-free medium, 0.812. These figures show that the chlorophyll-a content is significantly greater in cultures containing streptomycin than in those lacking streptomycin. It is also higher in the resistant cells when they are grown in a streptomycin supplemented medium than when grown in basal medium even at different phases of growth, e.g., the 4th, 8th or 12th day following inoculation (Table 3).

These results are also indicative of a possible *partial dependence* of the resistant strain on streptomycin for pigment synthesis. Fig. 7 further suggests that with the increase in age of cultures of both strepto-

Table 3. *Chlorophyll-a content (mg/2 × 10^8 cells) of control strain and of streptomycin-resistant strain grown in the presence or absence of streptomycin*

Age of cells in days	Control strain	Resistant strain	
	Basal medium	Basal medium	Basal + 50 μg streptomycin/ml
4	1.508	0.580	0.812
8	0.818	0.341	0.409
12	2.262	0.812	1.334

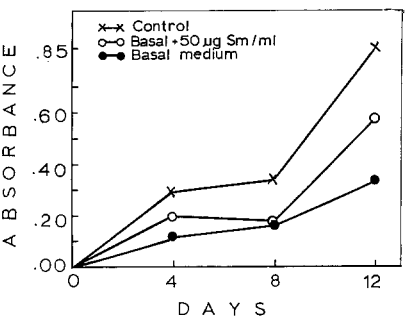

Fig. 7. Amounts of chlorophyll-a and carotenoids (430 nm) in pigment extracts from approximately equal cell lengths of induced streptomycin-resistant strain in basal medium, basal plus 50 μg streptomycin/ml., and control cultures

mycin-resistant strain and parent strain, there occurs an increase in the quantity of the pigments absorbing at 430 nm.

8. Effect of Proflavine Treatment on Pigmentation

Streptomycin-resistant cells were inoculated into a culture flask containing 0.1 μg proflavine/ml. After a 10 day growth in proflavine, cells were centrifuged and washed in sterile water thrice so as to remove all traces of proflavine. Washed cells were then inoculated into a culture flask supplemented with 50 μg streptomycin/ml and into another containing only basal medium. Acetone soluble-pigments were extracted on the 20th day following inoculation. Fig. 8 shows that there is no significant effect of proflavine treatment on the absorption maxima of either carotenoids or chlorophyll-a, and that the resistant strain still shows a partial dependence on streptomycin.

9. Attempts to Produce Penicillin-Resistant Strains by Diethyl Sulphate

Besides streptomycin-resistance, penicillin-resistant mutants were also sought after diethyl sulphate treatment. Cells were treated with

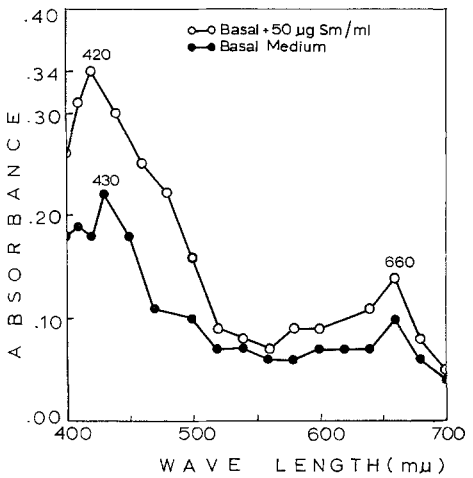

Fig. 8. Absorption spectra of acetone extracts of approximately equal cell lengths of induced streptomycin-resistant strain after treatment with 0.1 µg proflavine/ml, in basal medium and in basal plus 50 µg streptomycin/ml

0.1 M diethyl sulphate at pH 5.0 for 15 min. Treated and untreated cells were inoculated into a duplicate set of culture tubes supplemented with different concentrations of penicillin (0.001, 0.01, 0.05, 0.1 and 0.5 µg/ml). Growth of treated cells occurred after 2, 3, 5, 9 and 10 days of lag-period in concentrations of up to 0.5 µg/ml whereas the growth of untreated cells never occurred in concentrations greater than 0.09 µg/ml.

This experiment was repeated twice with invariably the same results thus suggesting that the 5 times higher resistance of diethyl sulphate treated cells over that of untreated controls might have resulted from the diethyl sulphate treatment.

Discussion

In the present study a streptomycin-resistant strain was isolated following treatment with the alkylating agent diethyl sulphate. This resistant strain was initially selected in 0.5 µg streptomycin/ml and the strain so obtained on further testing was found to be resistant to 50 µg streptomycin/ml. The fact that resistance is not lost even after several subcultures of the resistant strain in antibiotic-free basal medium strongly suggests that the resistance is stable. The application of modified Luria-Delbrück (1943) fluctuation analysis on the streptomycin-resistant strain revealed that the strain is a mutant strain induced as a result of diethyl sulphate treatment.

In the present study, treatment of *A. nidulans* with diethyl sulphate induced a 1000-fold increased resistance to streptomycin in a single step. It was observed that diethyl sulphate induced 50 μg streptomycin/ml-resistant strain further mutated to the level of 100 μg/ml and later to 500 μg/ml. This latter strain is thus about 10,000 times more resistant to streptomycin than the parent strain. These results indicate that diethyl sulphate induced streptomycin-resistant strain can further mutate to higher levels of resistance.

In bacteria, the proportion of antibiotic- and radiation-resistant mutants is usually increased after treatment with mutagenic agents (Northrop and Cavallero, 1961). In *Anacystis nidulans*, an ultraviolet-trained strain was found to be more resistant to streptomycin and penicillin than its untrained strain (Kumar, 1963). This fact was further confirmed by Kumar (1968) when he found that treatment with N-methyl-N′-nitro-N-nitrosoguanidine or ethyl methane sulphonate led to an increase in the proportion of streptomycin- and ultraviolet-resistant mutants. Kumar (1964) produced a streptomycin-resistant strain of *A. nidulans* which was 50,000 times more resistant to streptomycin than the untreated strain. He proceeded to test the nature of the streptomycin-resistant strain by using a modified technique of Luria and Delbrück (1943) and found that the resistant strain had arisen spontaneously. In the present study, the maximum level of streptomycin-resistance attained by the diethyl sulphate treated strain was approximately 10,000 times greater than that of the untreated strain, of which a factor of $\times 1000$ was induced by diethyl sulphate treatment and the remaining $\times 10$ was probably due to spontaneous mutations in two successive steps. The maximum concentration tolerated by the resistant strain in both Kumar's work (1964) and in the present study is found to be 500 μg streptomycin/ml.

The production of filaments following treatment with mutagenic or other toxic agents has been reported in bacteria (Kalle *et al.*, 1965; Greenberg *et al.*, 1961; Deering, 1959) and in blue-green algae (Kumar, 1963; Van Baalen, 1965; Singh *et al.*, 1966). The diethyl sulphate induced streptomycin-resistant strain of *Anacystis nidulans* also shows profuse filamentation during its growth in streptomycin-supplemented medium but no such effect is obtained in the parental, sensitive strain under similar conditions. Since the alga suffers two successive treatments, the diethyl sulphate and the streptomycin, the point in question is whether the observed filamentation is due to diethyl sulphate treatment or streptomycin treatment or both. The failure of streptomycin to induce filamentation in *A. nidulans* (Kumar, 1964) and the similar behaviour of the parental strain of this organism towards streptomycin in the present case suggests that the observed filamentation has primarily re-

sulted from diethyl sulphate treatment. Furthermore, the observation that the degree of filamentation in diethyl sulphate treated cultures decreases with increase in concentration of streptomycin strongly argues against any role of streptomycin in the induction of filamentation. Also, the possibility that diethyl sulphate treatment, in addition to causing mutation from streptomycin-sensitivity to streptomycin-resistance, might also have caused another mutation from non-filamentation to filamentation cannot be entirely ruled out at this stage. In this respect, a clear understanding of the nature of filamentation processes (e.g., whether reversible or non-reversible) would be of immense value and this can reasonably be ascertained provided the diethyl sulphate induced cultures are studied clonally during their growth on basal medium.

The induced resistant strain (50 μg streptomycin/ml) when inoculated in streptomycin-supplemented basal medium (50 μg/ml) and in basal medium, exhibited higher carotenoid- and chlorophyll-a content in the former than in the latter medium suggesting thereby a partial dependence of the resistant strain on streptomycin for pigment synthesis. It was further observed that proflavine treatment does not alter the phenomenon of partial dependence. This is suggestive of the fact that the observed dependence may in part be nuclear in character.

In a comparative study of the response of diethyl sulphate induced streptomycin-resistant strain and the parental strain to ultraviolet, it was observed that the induced resistant strain shows a comparatively shorter lag than the parental strain and grows in doses of ultraviolet much higher than those permitting the growth of parental strain. This obviously suggests that streptomycin-resistant strain is concomitantly resistant to ultraviolet killing. Previous studies on ultraviolet sensitivities of bacteria have clearly shown that ultraviolet induced lag is a direct function of the degree of ultraviolet-induced inhibition of DNA synthesis and that greater survival is a function of the operation of an efficient repair system that copes with the lethal lesions produced in the DNA of the irradiated organism (Hanawalt, 1966). Singh (1968) studied the nature of the factor controlling ultraviolet-sensitivity of three strains of *A. nidulans* (Singh's strain) and proposed that the degree of ultraviolet killing is a function of the genetically controlled magnitude of repair system that cures irradiated DNA and the generated pyrimidine dimers. If this be so in the present case, then diethyl sulphate treatment might conceivably have caused yet another mutation, namely, from radiation-sensitivity to radiation-resistance.

The streptomycin-resistance character as obtained in the present investigation seems polygenic as in our previous work (Kumar, 1964). The induction of different levels of streptomycin-resistance and penicillin-resistance in blue-green algae by alkylating agents could reasonably be

explained provided one accepts the view that the mechanism of action of alkylating agents is similar in all biological systems and that alkylating agents produce mutations by deletions and base changes.

In conclusion it may be stated that in the blue-green alga *Anacystis nidulans* diethyl sulphate seems to have produced a composite mutant strain, comprising at least three different kinds of mutations, namely, streptomycin-resistance, filamentation, and ultraviolet-resistance. In addition, the partial requirement for streptomycin may represent a possible fourth kind of mutation induced by diethyl sulphate.

Acknowledgments. We thank Dr. H. N. Singh for valuable discussions. This work forms part of Ph. D. thesis submitted by RSG under the guidance of HDK.

References

Alderson, T.: Ethylation versus methylation in mutation of *Escherichia coli* and *Drosophila*. Nature (Lond.) **203**, 1404—1405 (1964).

Allen, M. M.: Simple conditions for growth of unicellular blue-green algae on plates. J. Phycol. **4**, 1—4 (1968).

Auerbach, C.: The chemical production of mutations. Science **158**, 1141—1147 (1967).

Deering, R. A.: Radiation sensitivity of filamentous *Escherichia coli* Biochim. biophys. Acta (Amst.) **31**, 9—11 (1959).

Greenberg, J., Mandell, J. D., Woody, P. L.: A preliminary report—Resistance and cross-resistance of *Escherichia coli* mutants to radiomimetic agents. Cancer Chemother. Reports, No. 11, 51—56 (1961).

Hanawalt, P. C.: The u.v. sensitivity of bacteria: its relation to the DNA replication cycle. Photochem. Photobiol. **5**, 1—12 (1966).

Hughes, E. D., Gorham, P. R., Zehnder, A.: Toxicity of a unialgal culture of *Microcystis aeruginosa*. Canad. J. Microbiol. **4**, 225—236 (1958).

Kalle, G. P., Agrawal, A. K., Subramanian, S.: Mutation to u.v. resistance and filament formation in bacteria. Proc. 3rd Internat. Sympos., Ind. J. Genetics & Plant Breeding **26 A**, 431—440 (1965).

Kratz, W. A., Myers, J.: Nutrition and growth of several blue-green algae. Amer. J. Bot. **42**, 282—287 (1955).

Kumar, H. D.: Apparent genetic recombination in a blue-green alga. Nature (Lond.) **196**, 1121—1122 (1962).

— Effects of radiations on blue-green algae. I. The production and characterization of a strain of *Anacystis nidulans* resistant to ultraviolet radiation. Ann. Bot. **27**, 723—733 (1963).

— Streptomycin- and penicillin-induced inhibition of growth and pigment production in blue-green algae and production of strains of *Anacystis nidulans* resistant to these antibiotics. J. exp. Bot. **15**, 232—250 (1964).

— Effects of certain toxic chemicals and mutagens on the growth of the blue-green alga *Anacystis nidulans*. Canad. J. Bot. **43**, 1523—1532 (1965).

— Action of mutagenic chemicals on *Anacystis nidulans*. II. Ethyl methane sulfonate. Beitr. Biol. Pflanzen. **45**, 161—170 (1968).

— Action of mutagenic chemicals on *Anacystis nidulans*. III. N-methyl-N′-Nitro-N-nitrosoguanidine. Arch. Mikrobiol. **63**, 95—102 (1968).

Luria, S. E., Delbrück, M.: Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**, 491—511 (1943).

Northrop, J. H., Cavallero, F.: Mutagens and infectious nucleic acids. Proc. nat. Acad. Sci. (Wash.) **47**, 1602—1606 (1961).

Singh, H. N.: Effect of acriflavine on ultraviolet sensitivity of normal, ultraviolet-sensitive and ultraviolet-resistant strains of a blue-green alga, *Anacystis nidulans*. Radiat. Bot. 8, 355—361 (1968).

Singh, R. N., Sinha, R.: Genetic recombination in a blue-green alga *Cylindrospermum majus* Kuetz. Nature (Lond.) **207**, 782 (1965).

— Singh, H. N., Sinha, R.: Mutation and recombination in blue-green algae. Indian J. Genetics & Plant Breeding **26A**, 405—430 (1966).

Shestakov, S. V., Jevener, V. D.: Study of mutagenesis in blue-green alga *Anacystis nidulans*. Proc. XII Internat. Congr. Genetics, 1 (Abst.), 84 (1968).

Van Baalen, C.: Mutation of the blue-green alga *Anacystis nidulans*. Science **149**, 70 (1965).

Whatley, F. R., Arnon, D. I.: Photosynthetic phosphorylation in plants. In: Colowick, S. P., and N. O. Kaplan (eds.): Methods in Enzymology, vol. 6, pp. 308—313. New York: Academic Press 1963.

Professor H. D. Kumar
Department of Botany
Maharana Bhupal College
University of Udaipur
Udaipur, Rajasthan, India