Effects of Radiations on Blue-green Algae

II. Effects on Growth

BY

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With six Figures in the Text

ABSTRACT

The unicellular blue-green alga *Anacystis nidulans* was repeatedly treated with X-rays and radiophosphate (^{32}P) during successive subcultures. The strains so obtained were characterized by comparison with the untreated control strain, with respect to their resistance to ultraviolet light, X-rays, streptomycin, and isoniazid. The ^{32}P-treated strain was found to be relatively more resistant to streptomycin than the untreated strain and the X-rayed strain was found to be relatively more sensitive to isoniazid than the control. In old cultures, cells of the X-rayed strain were significantly smaller than those of the untreated strain.

The effects of X-rays on cell- and heterocyst dimensions of the nitrogen-fixing alga *Chlorogloea fritschii* Mitra were studied. In the irradiated material the cell diameter and heterocyst breadth were greater, rather than smaller, than in unirradiated material.

INTRODUCTION

EXPERIMENTS carried out by many workers have shown that, in general, algae are much more resistant to ionizing radiations than are higher plants. A few species of blue-green algae have hitherto been irradiated with X-rays with a view to studying their resistance or obtaining mutant forms (cf. Bonham and Palumbo, 1951; Singh, 1957), but apart from the work of Godward (1962), the use of radioactive isotopes such as ^{32}P for purposes other than that of tracing of metabolic pathways does not seem to have been reported for any blue-green alga.

Both X-rays and β-radiation from ^{32}P are proven and powerful mutagenic agents, the energy of the latter radiation being as high as 1·7 MeV. Radiophosphate also has a conveniently short half-life of 14·2 days and decays at a rate of 4·9 per cent per day, becoming transmuted to sulphur (^{32}S) which is stable and non-toxic. Breaks in the genetic material induced by ^{32}P incorporated into the DNA of *Escherichia coli* have been shown to be mutagenic (Kaudewitz, Vielmetter, and Friedrich-Freksa, 1958) and a significant increase in the mutation frequency of ^{32}P-treated cultures of a *Mycobacterium* has been reported by Tsukamura (1961).

The present investigation was designed to study the effects of X-rays and β-radiation from ^{32}P on the growth and survival of pure cultures of two species

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of blue-green algae, generally known as *Anacystis nidulans* and *Chlorogloea fritschii*, and to find out whether or not mutant strains of these algae could be isolated following these treatments. Specifically, the mutations sought were those affecting resistance to streptomycin and to isoniazid. Results obtained by treatment of *Anacystis nidulans* with ultraviolet light have been described in a previous paper (Kumar, 1963).

Materials and Methods

Strains

1. *Anacystis nidulans.* The strain of *A. nidulans* used in this study was the same as described in detail in previous papers (Kumar, 1963, 1964).

2. *Chlorogloea fritschii. C. fritschii* was isolated by Mitra (1950) from an Indian soil and described as a new species belonging to the Entophysalidaceae, order Chroococcales. A unialgal culture of this alga was obtained from the Cambridge Culture Collection of Algae and Protozoa and rendered bacteria-free by Dr. P. Fay (cf. Fay and Fogg, 1962). It seems that this alga is not, in fact, a member of the Chroococcales but, probably, an anomalous *Nostoc* (Fay, Kumar, and Fogg, 1964).

Culture Media and Methods

1. *Anacystis nidulans. A. nidulans* was grown in a slightly modified Medium C of Kratz and Myers (1955) as described in the previous papers.

2. *Chlorogloea fritschii.* This alga was grown in a slightly modified *Anabaena*-medium of Fogg (1949), diluted to half-concentration to minimize precipitation, and having the following composition in grammes per litre of pyrex-distilled water: K_2HPO_4, 0·10; $MgSO_4.7H_2O$, 0·10; $CaCl_2$, 0·025; NaCl, 0·025; $FeCl_3.6H_2O$, 0·0002; and 0·5 ml of an A_5 micronutrients stock solution as described in the previous papers. The pH of the culture medium was adjusted to approximately 8·0.

Culture flasks and pipettes were plugged with non-absorbent cotton-wool and autoclaved at 15 lb/square inch for 15 minutes.

Antibiotics

Streptomycin was supplied as sulphate (Glaxo, potency 745 units/mg), penicillin as benzylpenicillin, sodium salt (Glaxo, potency 1670 units/mg), and isoniazid (isonicotinic acid hydrazide) was a British Drug Houses product. Stock solutions were prepared in pyrex-distilled water, sterilized by Seitz filtration, and appropriate dilutions added to cold, sterile medium (equal amounts of water being added to controls). Aseptic precautions were taken throughout.

Irradiation Techniques and Production of Strains

Anacystis nidulans

(i) *X-rays.* In each of three Petri-dishes (5-cm diameter) were put 3 ml of a 24-hour-old suspension of the alga containing 5×10^6 cells/ml. Each Petri-dish was then covered with a sheet of thin 'Styrafoil-S', which lets most

of the radiation pass through it (Barclay, 1951, as quoted by Cosslett and Nixon, 1960) while at the same time ensuring asepsis during irradiation. The styrafoil sheets had previously been sterilized by exposure on both sides to ultraviolet light for 30 minutes and were held in place on the Petri-dishes by means of rubber bands. The suspensions covered in this manner were then exposed to X-rays at a distance of 8·5 cm. The characteristics of the radiation used were: 33 kV_p, 25 mA, 0·275 mm Al filtration, 3·1 mm wax half value thickness, dose rate 4,250 r/min. The total doses given were respectively 51,000, 85,000, and 102,000 r units. Inocula containing 10^6 cells of the irradiated suspensions were transferred to duplicate flasks containing 50 ml of basal medium and incubated at $31 \pm 5°$ C. Growth occurred in all the six flasks. After three weeks a suspension containing 5×10^6 cells/ml was prepared from the 102,000 r-irradiated culture and exposed to a second dose of 150,000 r. The third serial dosage was 204,000 r. At the fourth irradiation the characteristics of the radiation used were: 50 kV_p, 20 mA, 0·285 mm Al filtration, dose rate 3,713 r/min at a distance of 8·5 cm, and the total dose was 200,502 r. The fifth serial irradiation and dosage were similar to the fourth. The intervals between successive irradiations varied from ten days to three weeks. The strain thus obtained was designated as the X-rayed strain.

The X-rayed strain was given two subcultures, without treatment, in basal medium, each lasting 24 hours. These subcultures were grown in the culture apparatus described by Fogg, Smith, and Miller (1959) and maintained at a temperature of 39° C. The second subculture was the source of inoculum for starting experiments.

(ii) *β-radiation.* To flasks containing sterile basal medium was added aseptically, phosphorus-32 (as orthophosphate in dilute hydrochloric acid, solution sterilized, specific activity greater than 1,000 c/g P, supplied by the Radiochemical Centre, Amersham) at concentrations of about 0·001 mc/100 ml and 0·01 mc/100 ml. About 10^6 cells from a 24-hour culture were introduced into the flasks which were then covered with a bell-jar and illuminated by a 60-watt tungsten lamp. Growth occurred in both the flasks. From the culture in 0·01 mc/100 ml, a small inoculum was transferred after three weeks into a fresh flask containing 0·1 mc radiophosphate/100 ml. Further serial treatments of the alga with ^{32}P at concentrations of 0·1–0·3 mc/100 ml, were carried out at intervals of 2–3 weeks each. Five serial treatments were given. One month after the last treatment the alga was subcultured in basal medium and allowed to grow for one further month after which it was once again subcultured into the basal medium. After another month the radioactivity in this flask was found to have fallen to the background level. The culture so obtained was designated as the '^{32}P-treated strain'. In preparation for experiments inoculum was prepared from it in the same way as for the X-rayed strain.

X-irradiation of C. fritschii

A 5-cm Petri-dish containing 3 ml suspension from a 10-day-old culture of *C. fritschii* was covered with sterilized styrafoil-S and irradiated with

102,000 r of 33 kV_p X-rays at a distance of 8·5 cm. At the second and the third treatment the dosage was increased to 150,000 r. At the fourth and the fifth irradiation the dosage given was 150,000 r but the characteristics of the radiation used were: 50 kV_p, 20 mA, 0·285 mm Al filtration with a dose rate of 3,713 r/min at a distance of 8·5 cm. The intervals between successive irradiations varied from ten days to three weeks. Cultures were maintained at a temperature of $31 \pm 3°$ C and the light intensity was 350 ± 50 f.c.

Results

Characterization of Treated Strains of A. nidulans

Resistance to X-rays. The X-rayed strain was irradiated with 0, 1×10^5, 2×10^5, and 3×10^5 r of 50 kV_p X-rays, the Petri-dishes containing 3 ml of

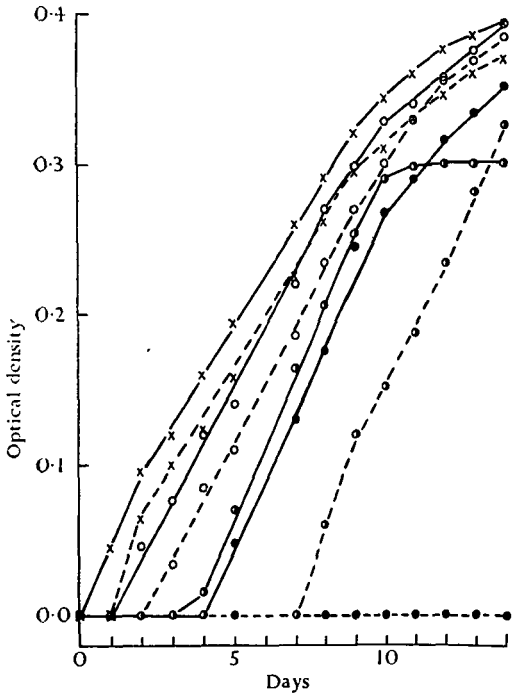

Fig. 1.

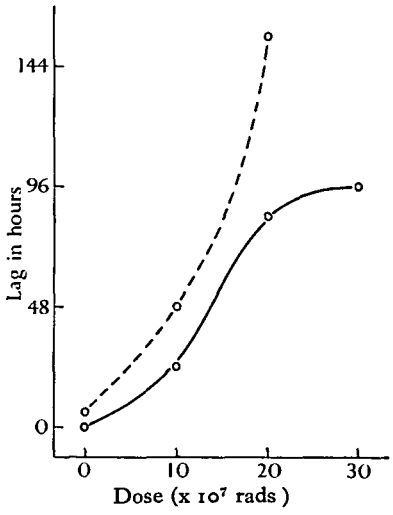

Fig. 2.

Fig. 1. *A. nidulans.* Resistance to X-rays of the X-rayed strain. ○ 100,000 r, ◑ 200,000 r, ● 300,000 r, × unirradiated.
—— Inoculum 10^6 cells ..... Inoculum 10^5 cells

Fig. 2. *A. nidulans.* Resistance to X-rays of the X-rayed strain. X-ray dosage-lag relations.
—— Inoculum 10^6 cells. .....Inoculum 10^5 cells.

suspension (10^7 cells/ml). After each dosage, 10^5 and 10^6 cells each from appropriate dilutions in water of the irradiated material were transferred into

duplicate culture tubes containing 20 ml of medium. The tubes were incubated in the culture tank at 39° C and shaken once daily.

Growth occurred in all tubes inoculated with 10^6 cells (Fig. 1). In those that had been inoculated with 10^5 cells growth occurred in all except the last two containing 3×10^5 r-irradiated cells. The dosage-lag relations observed are plotted in Fig. 2.

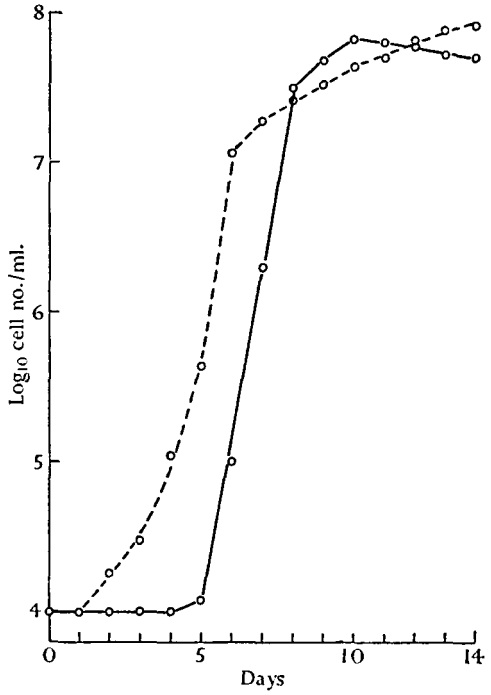

FIG. 3. *A. nidulans*. Comparison of resistance to ultraviolet light of X-rayed and untrained strains.

—— untrained strain, 15-min. UV-irradiated.
..... X-rayed strain, 15-min. UV-irradiated.

Resistance to ultraviolet light and distribution of cell lengths. The X-rayed strain was compared with the 'untrained' parent strain for resistance to ultraviolet light according to the procedure described in a previous paper (Kumar, 1963). With the inoculum size used, growth was found to have occurred in only those flasks that had been inoculated with material irradiated for up to 15 min. Thus, unlike the ultraviolet-resistant strain (Kumar, 1963), the X-rayed strain did not manifest increased tolerance to ultraviolet light as compared to the untrained, parent, strain. The growth of the 15-min-irradiated X-rayed strain was, however, better than that of the untrained strain both as regards the duration of the lag-phase and the final population density (Fig. 3).

Samples of about 100 cells of the 15-min-irradiated X-rayed strain and of the untrained, parent, strain were measured at the start of the experiment and then again on the 7th and the 14th day. Initially, 69 per cent of the cells of the X-rayed strain were less than 3·99 μ long (Fig. 4) whereas in the untrained, parent strain, 90 per cent of the cells fell in this category. On the 7th day, the percentage of such cells increased to 76 in the X-rayed strain but decreased to 79 in the controls, the mean cell lengths of the two strains not

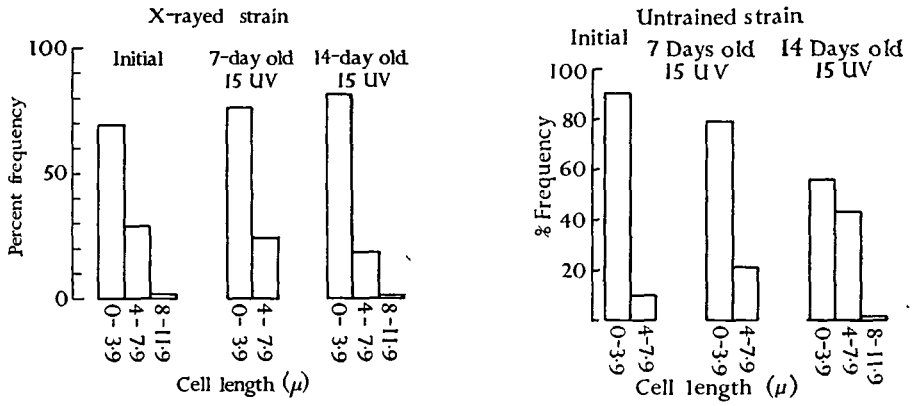

FIG. 4. *A. nidulans.* Histograms of frequency distribution of cell lengths of ultraviolet-irradiated material of X-rayed and untrained strains.

differing significantly (Table 1). In 14-day-old cultures the proportion of cells shorter than 3·99 μ increased still further to 81 per cent in the X-rayed strain and decreased to 56 per cent in the untrained strain, resulting in a significant decrease in the mean cell length of the X-rayed strain as compared with that of the untrained. This is in contrast to the results obtained with the ultraviolet-resistant strain of *A. nidulans* (cf. Kumar, 1963) whose cells were significantly longer than those of the untrained strain. Comparison between the X-rayed strain and the ultraviolet-resistant strain is, however, not strictly justified in view of the fact that the former was derived by only five successive treatments in contrast to 25 for the latter.

TABLE 1

Significance of Difference between Mean Cell Lengths of X-rayed and Untrained Strains of A. nidulans *after 15-min Ultraviolet-irradiation*

	Difference between means (μ)	Standard error of difference	Probability
Initial	1·09	0·195	$< 0·001$
7-day old	0·08	0·170	$> 0·60$
14-day old	1·21	0·173	$< 0·001$

Resistance to streptomycin. A series of duplicate flasks containing streptomycin at concentrations of 0·001, 0·002, 0·004, and 0·006 mg/100 ml, were

inoculated with approximately 10^6 cells each from the X-rayed strain. A second batch of similar flasks were inoculated with 10^6 cells each from the ^{32}P-treated strain, and a third batch was similarly inoculated with the untrained strain. The flasks were incubated in the culture tank at 39° C and shaken once daily. All flasks containing 0·001 mg streptomycin/100 ml revealed growth which was almost the same in the three strains. In addition,

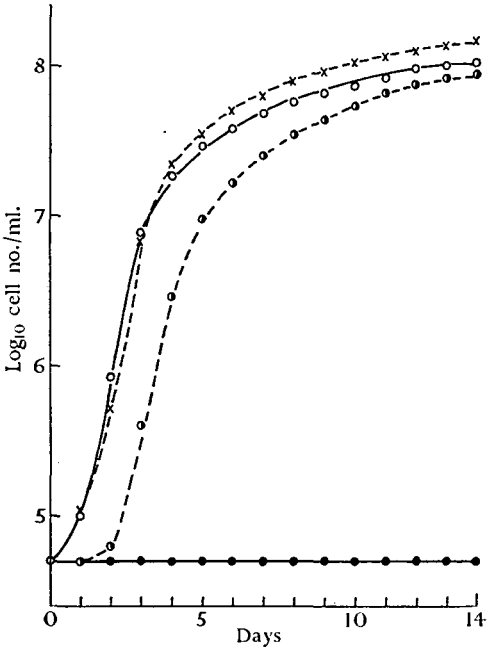

FIG. 5.

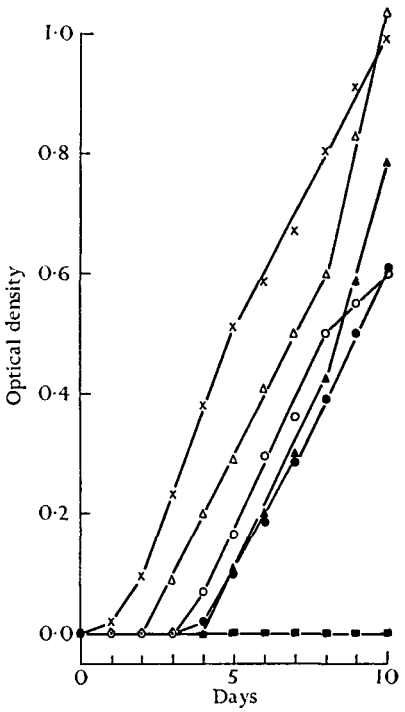

FIG. 6.

FIG. 5. *A. nidulans.* Comparison of resistance to streptomycin of untrained and ^{32}P-treated strains. —— untrained strain. ..... ^{32}P-treated strain. ○ and × 0·001 mg streptomycin/100 ml.; ◑ and ● 0·004 mg streptomycin/100 ml.

FIG. 6. *A. nidulans.* Comparison of resistance to isoniazid of untrained, X-rayed and ^{32}P-treated strains. ○ untrained strain in 1 mg isoniazid/100 ml; ● untrained strain in 2 mg isoniazid/100 ml; △ ^{32}P-treated strain in 1 mg isoniazid/100 ml; ▲ ^{32}P-treated strain in 2 mg isoniazid/100 ml; × X-rayed strain in 1 mg isoniazid/100 ml; and ■ X-rayed strain in 2 mg isoniazid/100 ml.

growth occurred in four flasks containing 0·002 and 0·004 mg streptomycin/100 ml and inoculated with the ^{32}P-treated strain (Fig. 5) after a lag of about two days, the same population density being attained on the 14th day as that attained by the untrained strain in 0·001 mg streptomycin/100 ml.

Resistance to isoniazid. The X-rayed strain, like the ultraviolet-resistant strain, was found to be more sensitive to isoniazid than the untrained strain. Unlike the ultraviolet-resistant strain, however, the X-rayed strain could

grow in concentrations of isoniazid up to only 1·0 mg/100 ml, the maximum concentration tolerated by the untrained strain being 2·0 mg/100 ml (Fig. 6).

The isoniazid-resistance of the ^{32}P-treated strain was about the same as that of the untrained parent strain, although the former grew after a slightly longer lag-period (Fig. 6).

Effects of X-rays on Growth of C. fritschii

The effects of 150,000 r of 33 kV_p X-rays on cell diameter, heterocyst length, and heterocyst breadth of the alga were studied by inoculating irradiated and unirradiated material of known initial measurements into: (1) basal nitrogen-free medium, as already described; (2) basal+1 per cent glucose; (3) basal+1 per cent glucose+0·05 per cent yeast extract (Bacto); (4) basal+0·5 per cent casamino acids (vitamin-free); (5) basal+0·1 ml per cent of a vitamin-mix stock solution containing the following vitamins in mg/100 ml distilled water: *d*-biotin, 0·2; riboflavin, 50; *p*-aminobenzoic acid, 10; nicotinamide, 100; pyridoxal hydrochloride, 50; *d*-pantothenic acid, calcium salt, 100; *i*-inositol, 400; aneurine hydrochloride, 50; and folic acid, 10; and (6) basal+1 mg per cent of each of adenine, thymine, guanine, and uracil. The suspensions irradiated were from 7-day-old cultures. The inoculated flasks were maintained in a culture chamber at 33±3° C and 350±50 f.c., and shaken once daily.

There was no growth in flasks that had been supplemented with nucleic acid bases. In casamino acids growth was very poor and the cells appeared granular, lysed, and degenerate. In the other supplements, growth was about the same as in unsupplemented medium. From 1-month-old cultures about 40–60 cells and heterocysts, chosen at random, were measured in each case but no very pronounced differences were found. Cell diameter and heterocyst breadth were significantly greater in the irradiated material grown in basal medium than in the corresponding unirradiated material and a statistically significant decrease in heterocyst length was observed in the X-rayed material inoculated into the medium supplemented with vitamins.

The results of a comparison of the nitrogen-fixing capacities of irradiated and unirradiated material of *C. fritschii* are given elsewhere (Fay, Kumar, and Fogg, 1964).

Discussion

The results obtained by the X-ray treatment of *Anacystis nidulans* and *Chlorogloea fritschii* support the view that blue-green algae are much more resistant to ionizing radiations than are other algae or higher plants (Table 2).

An interesting finding of the present investigation is that in old cultures the mean cell length of the X-rayed strain of *A. nidulans* was significantly smaller than that of the untrained strain. This may be due to some stimulatory effect of the radiation on the cell-division mechanism of this alga. However, this type of effect was not found in *C. fritschii*.

TABLE 2

Comparison of Sensitivity to X-rays of Certain Algae and Other Organisms

Organism	Approximate lethal dose (rads)	Reference
Chlamydomonas reinhardtii	10×10^3	Nybom (1953)
C. moewusii	$15–20 \times 10^3$	Guillard (1960) p. 262
Pandorina morum	$5–300 \times 10^3$	Halberstaedter and Back (1942)
Oedogonium cardiacum zoospores	750 (for 50% death)	Howard and Horsley (1960)
Spirogyra crassa	15×10^3 (for 'ultimate survival')	Godward (1962)
Synechococcus sp.	100×10^3 (for survival)	Bonham and Palumbo (1951)
Anacystis nidulans	$100–300 \times 10^3$ (or more)	(This paper)
Saccharomyces cerevisiae Strain SC 7	$10–100 \times 10^3$	Alper (1959)
Hordeum distichum (seminal roots)	$2{\cdot}5 \times 10^3$	Ebert and Barber (1961)

No great resistance to streptomycin was found in the ^{32}P-treated strain of *A. nidulans*. Like the ultraviolet-resistant strain, it showed only a small increase in streptomycin resistance, by about four times, as compared to that of the untrained strain.

According to Tsukamura (1961), growth of *Mycobacterium avium* in a culture medium containing ^{32}P (0·1 mc/100 ml) can result in an increase in the proportion of isoniazid-resistant mutants by about eight times, and of those resistant to streptomycin by about 30 times, over the controls. In the present study although estimations of mutation frequencies were not made, it is obvious that marked increases in resistance to isoniazid and streptomycin were not manifested by the ^{32}P-treated strain of *A. nidulans*.

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