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The Effect of Maleic Hydrazide on Growth and Mutation of a Blue-Green Alga

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Summary. Maleic hydrazide is mutagenic to the blue-green alga *Anacystis nidulans* at pH 5.0, producing mutations to streptomycin-resistance and penicillin-resistance, and is non-mutagenic at pH 8.0, promoting growth in low concentrations. The maleic hydrazide-induced increases in the levels of streptomycin- and penicillin-resistance are 1000 and 500 times respectively over the parental strain. The resistant strains can further mutate spontaneously to give rise to strains resistant to still higher concentrations of the antibiotic.

Cultures growing with manganese are more sensitive to maleic hydrazide-induced inhibition of growth than are those growing without manganese. However, cultures of the alga in maleic hydrazide grown in the presence of two different concentrations of manganese differ in the extent of growth which is better in those with the higher manganese concentration than in those with the lower. These results suggest different modes of interaction between manganese and maleic hydrazide, controlled primarily by manganese concentration.

Maleic hydrazide (MH) produces a variety of physiological and genetical effects in many biological systems and, like other radiomimetic agents, has been observed to induce chromatic aberrations in mitotically dividing cells (Kihlman, 1961), to delay the onset of mitosis (see Scott, 1968) and to cause severe depression in the rate of DNA synthesis (Evans and Scott, 1964). Swanson and Kihlman (1956) studied its mutagenic efficiency at different pH values and found it to be strongly mutagenic at pH 5.0 and practically non-mutagenic at pH 7.0.

Besides mutagenic action, MH has been shown to inhibit overall growth of algae (Kim and Greulach, 1962), to cause reduction in the DNA and amino acid contents of *Chlorella* (Kim and Greulach, 1963) and to suppress respiration in the bacterium *Escherichia coli*, the suppression being reversed by cysteine, manganese, cobalt, zinc, nickel or iron (Suda, 1960). Tamiya *et al.* (1962), in a study of the effects of antimetabolites on growth and cell division of *Chlorella*, found MH to be a strong inhibitor of growth in high concentrations and an equally strong promotor of cell division in low concentrations.

Most of the above studies were confined to eucaryotic organisms and whether or not MH is also mutagenic to procaryotes has not yet been conclusively established. The present study was taken up with a view to studying the physiological and mutagenic effects of MH on a blue-green alga. Little, if any, work seems to have been reported hitherto on the effects of MH on Cyanophyta.

Material and Methods

The axenic and clonal strain of *Anacystis nidulans* used in this study, the general culture methods, composition of culture media, and culture conditions were the same as described in the accompanying paper (Gupta and Kumar, 1970). Methods of growth determination were also similar to those described previously.

The MH dose-response curve was obtained by treating algal suspension (4.5×10^6 cells/ml) with 100 mg MH/10 ml of citrate-phosphate buffer (pH 5.0). The algal suspension was added to the autoclaved MH solution of the above concentration. The treatment was carried out by shaking the flask on a mechanical shaker for 0, 10, 15, 30, 60 and 90 min. After washing thrice with sterile water, treated samples were plated on basal medium and plates incubated inverted in culture cabinets. After two-week incubation, percentage survival was scored by colony counting. Fig. 1 indicates that a dose of 90 min is lethal. Since the isolation and scoring of viable mutants requires a dose of mutagen that permits a sufficient number of treated cells to survive, accordingly a 30 min dose of MH at which survival was fairly high, was selected for the isolation of MH-induced streptomycin- or penicillin-resistant mutants (see Fig. 1).

Three-day old cells of *A. nidulans* were first concentrated by centrifugation to get about 3 ml thick suspension of inoculum (4.5×10^6 cells/ml). Two 150-ml conical flasks, each with 9 ml of citrate-phosphate buffer (pH 5.0), were taken and into one of these, 100 mg MH was added. Both the flasks were autoclaved and upon cooling, each was inoculated with 1 ml of centrifuged algal suspension and shaken on a flask shaker for 30 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. Samples (0.2 ml containing 2.8×10^6 cells) from the control and treated cultures were then inoculated into culture tubes (150×25 mm) containing 20 ml of medium and supplemented with appropriate concentrations of streptomycin and penicillin.

Results

Production of Streptomycin-Resistant Strain

Three-day old cells were treated with 100 mg MH/10 ml buffer (pH 5.0) for 30 min and inoculated into culture tubes containing basal medium supplemented with nil (control), 0.005, 0.05, 0.25 and 0.50 μg streptomycin/ml. Untreated cells were also inoculated in the above concentrations of streptomycin. Growth of treated cells occurred in all the concentrations of streptomycin used. In contrast, untreated cells grew only in the first three concentrations but not in the higher ones. Inocula (0.2 ml) from the population of treated cells in 0.5 μg streptomycin/ml were transferred into a duplicate set of culture tubes supple-

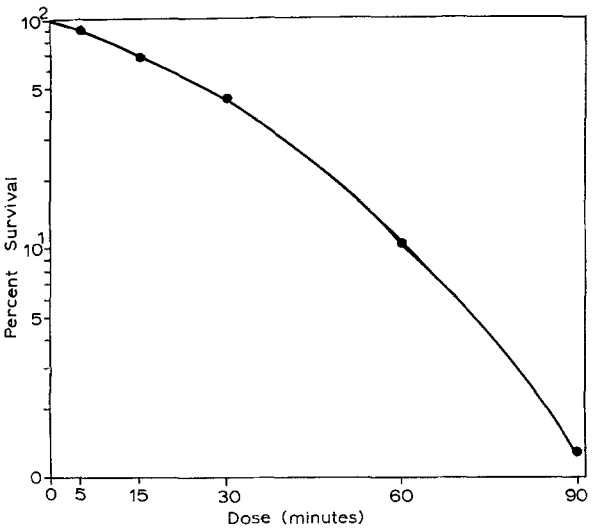

Fig. 1. Survival curve of *Anacystis nidulans* treated with maleic hydrazide

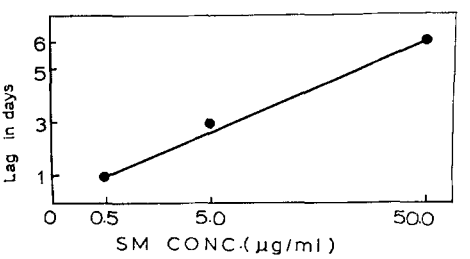

Fig. 2. Relationship between streptomycin concentrations and lag times following inoculation of a strain of *Anacystis nidulans* resistant to 0.5 µg streptomycin/ml into different concentrations of streptomycin

mented with 0.5, 5.0 and 50.0 µg streptomycin/ml. In these three concentrations growth occurred after 1, 3 and 6 days of lag-period respectively (Fig. 2). The treated cells grown in 0.5 µg streptomycin/ml showed abnormally long and coiled filaments on the 20th day. The lengths of the long coiled filaments and their percentage frequency in 0.5 µg streptomycin/ml in comparison to controls are shown in Fig. 3.

Cells growing in 50 µg streptomycin/ml were subcultured in streptomycin-free basal medium 5 times and then inoculated into 6 culture tubes each containing 50 µg streptomycin/ml. Growth occurred in all the 6 tubes, showing thereby that the streptomycin-resistance in this case was a stable trait.

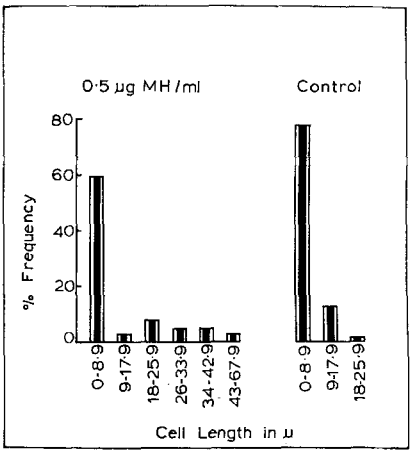

Fig. 3. Histograms of frequency distribution of cell lengths of maleic hydrazide treated cells of *Anacystis nidulans* in 0.5 µg streptomycin/ml and in basal medium

Growth of Resistant Strain in Higher Concentrations

Cells from the 50 µg streptomycin/ml-resistant strain (5×10^5 cells/ml), when inoculated in duplicate tubes containing 50, 100, 200 and 500 µg streptomycin/ml, never grew in any of the concentrations higher than 50 µg/ml. But when a series of 6 parallel samples, each of 2×10^5 cells/ml, from two different cultures of the resistant strain was taken and inoculated into 12 tubes containing 100 µg streptomycin/ml each, growth occurred in only one tube out of 12 after a lag of 12 days. When cells from this surviving culture were subcultured in the presence of 100 µg streptomycin/ml, growth occurred without a lag. The 100 µg streptomycin/ml-resistant strain so obtained was subcultured five times successively in the absence of streptomycin and then inoculated in media containing 100 µg streptomycin/ml. It was found that growth readily occurred and was preceded by virtually no lag. Since only one of the original 12 tubes containing 100 µg streptomycin/ml developed growth, it may be inferred that the 100 µg/ml-resistant strain arose spontaneously in one of the 50 µg resistant cultures and was then selected by the screening concentration. Moreover, the isolation of 200 µg/ml-resistant strain directly from the 50 µg/ml-strain was never successful and the 200 µg/ml-resistant strain could only be obtained from 100 µg/ml-strain by a method similar to the one adopted for isolation of 100 µg strain from 50 µg strain. This suggests that the 200 µg streptomycin/ml-resistant strain might have arisen from the 100 µg strain in the same way in which the 100 µg strain arose from 50 µg strain.

Table 1. *Showing growth characteristics of maleic hydrazide-treated and control cells of* A. nidulans *in different concentrations of penicillin*

Penicillin concentration (μg/ml)	MH-treated		untreated	
	Lag in days	O.D. on 14th day	Lag in days	O.D. on 14th day
0.0	1	2.0	1	2.1
0.005	1	1.9	1	1.9
0.05	6	1.8	5	1.9
0.25	7	1.9	—	—
0.50	8	1.9	—	—

(— indicates no growth).

In addition to streptomycin-resistance, penicillin-resistant strains were also scored following treatment of *A. nidulans* with maleic hydrazide. With other conditions remaining unaltered, the concentrations of penicillin used for selection of resistant strains were 0.005, 0.05, 0.25 and 0.5 μg penicillin/ml. Growth occurred in all the concentrations in treated cells; in the case of untreated controls, growth occurred only in the first two concentrations but not in the higher ones. Growth was followed by noting the lag-phase in each case and by the final optical density on the 14th day (Table 1).

The 0.5 μg penicillin/ml-resistant strain so obtained was inoculated into duplicate tubes containing 0.5, 5.0 and 50.0 μg penicillin/ml. Growth occurred in all these tubes following lag-phases of 8, 10 and 13 days respectively. A strain resistant to 50 μg penicillin/ml was obtained in this way.

4×10^5 cells each from the 50 μg penicillin-resistant strain were inoculated into duplicate tubes supplemented with 50, 100, 200 and 500 μg penicillin/ml. No growth was observed in concentrations higher than 50 μg/ml. Six parallel samples (2×10^5 cells/ml) from two different cultures of the 50 μg penicillin/ml-resistant strain were then withdrawn and each inoculated into a tube containing 100 μg penicillin/ml; growth occurred in only one tube out of 12, after a lag of 10 days. The inference drawn with respect to streptomycin-resistant strain would thus seem to apply in this case also.

Attempts to Adapt A. nidulans *to Higher Doses of MH*

Different concentrations of MH were added directly to the medium at pH 8.0 before autoclaving. Three-day old cells of the alga were inoculated in flasks supplemented with nil, 0.5, 1.0, 5.0, 10 and 20 μg MH/ml. Growth occurred in all the concentrations after a lag of 1—3 days.

Table 2. *Showing specific growth rates* (k) *of* Anacystis nidulans *in different concentrations of maleic hydrazide*

MH concentration (μg/ml)	*k*
0.0 (Control)	0.270
10.0	0.274
100	0.278
500	0.268

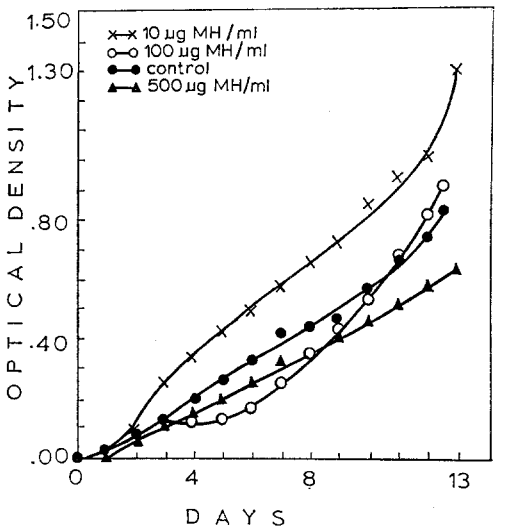

Fig. 4. Effect of maleic hydrazide on growth of *Anacystis nidulans*

In another experiment with higher concentrations of MH, i.e., 10, 20, 50 and 100 μg/ml, growth occurred in all the flasks after a lag of 1—3 days. The growth was better in 50 μg MH/ml and 100 μg MH/ml than in the controls. This indicates some stimulatory effect of MH on growth of the alga. To check this point further, specific growth rates in different concentrations of MH were determined and the values obtained (Table 2) indicate that MH promotes growth in low concentrations (Fig. 4).

To see whether or not the algal cells could be adapted to grow in concentrations of MH higher than those tolerated by parent cells, 10-day old cells from 500 μg MH/ml culture were inoculated into 500, 750 and 1000 μg/MH/ml and subcultured five times successively. No growth occurred in concentrations higher than 500 μg MH/ml.

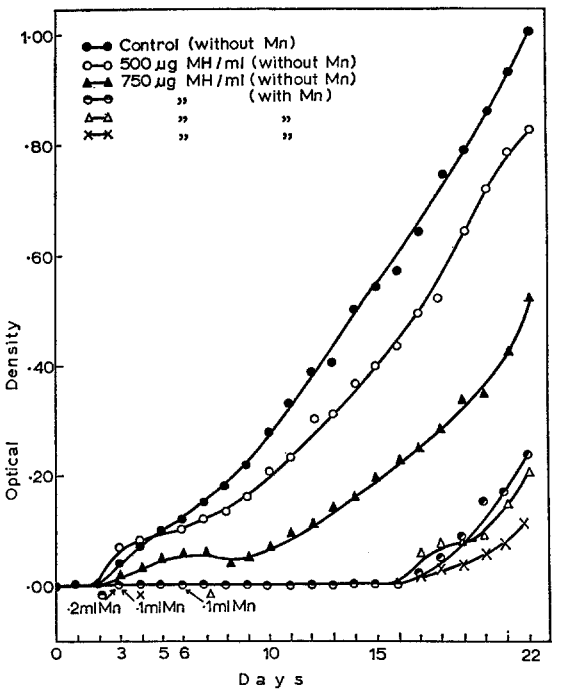

Fig. 5. Effect of maleic hydrazide and manganese on growth of *Anacystis nidulans*

Effect of MH and Manganese Chloride on Growth

To detect any antagonistic effect of MH and manganese chloride on the growth of *A. nidulans*, a set of 6 flasks was prepared, each with 100 ml of manganese-free medium. Into flask 1, 500 µg MH/ml (the highest concentration tolerated by the alga) was added; into flasks 2, 3, 4 and 5, 750 µg MH/ml was added, and the 6th flask without MH and without manganese was left as control. All 6 flasks were inoculated with 3-day old cells. Manganese chloride stock solution (0.1 ml) of the composition 1.80 gm per litre water was added on the 3rd and the 6th day into flasks 2 and 3 respectively. 0.2 ml of the manganese stock solution was added into flask 4 on the 3rd day and no manganese was added into flask 5.

Growth occurred in the first, fifth and sixth flask after a lag of 3 days in each. Flasks 1 and 5 with 500 and 750 µg MH/ml respectively showed less growth than flask 6 (control). On the 22nd day, growth in the fifth flask was just about one-half of that in the control. In the 2nd, 3rd, and 4th flask, growth appeared after a lag of 17 days and continued to rise upto the 22nd day. On the 22nd day, growth was better in the 3rd flask, wherein 0.1 ml of manganese stock was added on the 6th day than in the

second flask wherein the same amount of manganese was added on the 3rd day. However, better growth than these was noticeable in the fourth flask to which manganese was added in double the quantity (Fig. 5).

Discussion

The chemical basis of a mutagen is that it should react with DNA and the product of reaction should be a chemically altered DNA functionally different from the original one. Chemical alterations of DNA have been observationally divided into mutagenic and inactivating alterations. Both alterations can give rise eventually to cellular mutations but on the whole, inactivating alterations are more frequently lethal for the cells than mutagenic ones. Furthermore, the mechanism of mutation induction is quite different for the two alterations. Whereas mutagenic DNA alterations do not prevent DNA replication across the altered site, inactivating alterations invariably block DNA replication and the result of these two types of alterations is that the former generally leads to point mutations and the latter to mostly chromosomal breaks or large chromosomal aberrations. All the known mutagens bring about both types of alterations but they differ in respect of the relative proportion of the two alterations produced by them in the DNA.

MH produces mutations to streptomycin- or penicillin-resistance at pH 5.0 and stimulates growth in lower concentrations at alkaline pH. These results are in agreement with the observation of Swanson and Kihlman (1956) that MH is mutagenic in acid pH and non-mutagenic in alkaline pH. Furthermore, MH in addition to being mutagenic is also lethal since its 90 min dose kills almost all the treated cells (Fig. 1). Lethality results from the irreversible inactivation of DNA and a mutation is a consequence of either mutagenic alteration or inactivating alteration. The question as to which of these alternatives is applicable to MH-induced drug-resistant mutations is rather difficult to decide at this stage. Nevertheless, the mode of MH action seems to depend on pH as it causes mutation at pH 5.0 and promotes growth at pH 8.0.

The mechanism of MH action is not perfectly known. Leopold and Klein (1952) and subsequently others have proposed that MH is an anti-auxin, but a number of other investigators have produced evidence to the contrary. According to Isenberg *et al.* (1954) and Naylor and Davis (1949) MH inhibits respiration but it is doubtful whether growth inhibition is a result of this decrease in respiration. Others have suggested that MH inhibits growth by interfering with nucleic acid synthesis, particularly as regards the uracil component (see Kim and Greulach, 1963).

Like diethyl sulphate, MH initially confers in *A. nidulans* a 1000-fold higher resistance to streptomycin (see Gupta and Kumar, 1970). But the DES-induced streptomycin-resistant strain gave rise upon subsequent subcultures in basal medium to a streptomycin-resistant strain which was 10 times more resistant than the diethyl sulphate-induced strain and 10000 times more resistant than the parental strain. However, the MH-induced streptomycin-resistant strain increased its streptomycin-resistance level only four times following its subcultures in the basal medium. These observations clearly indicate that the diethyl sulphate and MH-induced strains differ in respect of their behaviour concerning spontaneous mutation to still higher levels of streptomycin-resistance. Further, whereas diethyl sulphate failed to induce high level penicillin-resistance in *A. nidulans*, MH treatment led to the production of strains that were 500 times more highly tolerant to penicillin than the parent strain. The MH-induced penicillin-resistant strain, like its streptomycin-resistant strain, mutates spontaneously to increase its level of penicillin resistance by a factor of two. The interesting point is why does MH induced streptomycin-resistance increase spontaneously to 4 times the initial level whereas the MH induced penicillin-resistance increases to only twice the original level? These results suggest that among mutagens there are different categories based on their affinity for different genes and that even a single mutagen capable of altering the function of a number of genes is likely to alter the function differentially. However, additional experimental data are needed to substantiate these propositions.

In alkaline pH, MH stimulates growth which seems to be a function of the MH concentrations. Higher concentrations of MH retard growth which does not occur in the basal medium containing more than 500 μg MH/ml. Manganese seems to increase the sensitivity of the alga to MH since growth occurs even with 750 μg MH/ml in the absence of manganese. Moreover, cultures with 750 μg MH/ml in which 0.1 ml of manganese solution is added after 6 days of inoculation show better growth than those in which the same amount of manganese is added after 3 days. In terms of apparent growth the timing of addition of manganese to the cultures does not seem significant since 750 μg MH/ml cultures supplemented with 0.1 ml manganese stock show a lag of approximately 17 days which appears to be independent of the timing of manganese addition. These observations are at variance with those wherein manganese is found to increase the sensitivity of the alga to MH. The better growth of 750 μg MH/ml cultures with 0.2 ml manganese solution than with 0.1 ml solution suggests some antagonistic effect of manganese against MH, but this again is contradictory to the observation wherein the absence of manganese enables *A. nidulans* to grow in concentrations of MH in which the alga fails to grow in the presence of manganese. Obviously,

much additional experimental data are needed to resolve this complex situation.

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