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Action of Mutagenic Chemicals on *Anacystis nidulans*

III. N-Methyl-N′-Nitro-N-Nitrosoguanidine

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Summary. The tolerance of the unicellular blue-green alga *Anacystis nidulans* to nitrosoguanidine increased from an initial level of 5 μg NTG/ml to 20 μg/ml during the course of 10 successive transfers in NTG-supplemented medium.

Single treatments of log-phase cells with NTG at pH 5—6 resulted in a significant increase in the frequency of streptomycin-resistant mutants but only a marginal increase in the frequency of ultraviolet-resistant mutants was observed following single or 10 successive treatments with NTG.

Attempts to score mutants resistant to penicillin, aminotriazole or NTG were unsuccessful since these agents failed to effectively kill or bleach the sensitive background population on the agar plates.

Following the first reports attributing a weak anti-tumor activity (GOLDIN *et al.*, 1959) and a mutagenic effect (MANDELL and GREENBERG, 1960) to N-methyl-N′-nitro-N-nitrosoguanidine (NTG), many papers confirming these effects and more especially the strongly mutagenic nature of NTG have been published from different laboratories. The organisms on which a potent mutagenic effect of NTG has been demonstrated include bacteria (ADELBERG *et al.*, 1965; EISENSTARK *et al.*, 1965), fungi (LOPRIENO and CLARKE, 1965; NORDSTRÖM, 1967), algae (GILLHAM, 1965; MCCALLA, 1967) and higher plants (MÜLLER and GICHNER, 1964). However, except for one report (VAN BAALEN, 1965) no previous studies on the action of this mutagen on any blue-green alga seem to have been carried out. VAN BAALEN (1965) was successful in producing mutations affecting various physiological and biochemical characeristics of *Anacystis nidulans* by treating it with NTG.

The aim of the present work was to study the physiological and mutagenic effects of NTG on the unicellular blue-green alga *Anacystis nidulans*, the mutations sought being those affecting resistance of the alga to streptomycin or to ultraviolet light. In previous papers (KUMAR, 1968a, 1968b) the effects of the alkylating mutagenic agents methyl- and ethylmethane sulfonate on this organism have been described.

Material and Methods

The strain of the alga and general methods for its culture and growth measurement were the same as in previous work (KUMAR, 1968a). The alga was grown in pure bacteria-free culture in ASM-1 medium (pH 8.6—8.9) of the composition previously described, in shake flasks. N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was purchased from Aldrich Chemical Co., Milwaukee, and used without further purification. For each experiment a fresh saturated stock solution of NTG (approximately 25 mg NTG/100 ml of cold sterile water) was prepared and appropriate volumes added to culture media to obtain the desired concentrations.

In preparation for mutation experiments, log-phase cells were harvested by Millipore filtration, washed with M/20 Tris-maleic buffer of pH 8.0 (ADELBERG *et al.*, 1965) and suspended in 20 ml of Tris-maleic buffer (pH 5.0). After spreading 0.2 ml or 0.5 ml aliquots from appropriately diluted suspensions from this on to ASM-1 agar plates (to serve as controls), freshly-prepared solution of NTG in tris-maleic buffer (pH 5) was added into the remaining suspension so as to get a final NTG concentration of about 400 μg/ml. The reaction mixture was constantly stirred on a magnetic stirrer during treatment. After 10 or 30 min, aliquots were withdrawn and cells washed twice with cold sterile ASM-1 (pH 8.6) on millipore filters. Washed cells were then suspended in ASM-1 to get the same cell concentrations as in untreated controls and 0.2 ml or 0.5 ml aliquots spread on ASM-1 agar plates. All plates were sealed with Parafilm and incubated at 22° C, 4,000—5,000 lux for 10 days. They were then either overlaid with a second layer of agar containing streptomycin (10 or 50 μg/ml) or exposed to ultraviolet light for 15 min (see KUMAR, 1968a), the colonies surviving the challenge being scored as mutants.

Results

Effect of NTG on Growth and Survival of A. nidulans *in Liquid Medium*

When the alga is inoculated into culture flasks containing NTG-supplemented medium, the maximum concentration of NTG permitting growth, after a lag of 6—5 days, is about 5 μg/ml (Fig. 1). Growth in such concentrations of NTG may, however, be due to the destruction or inactivation of NTG during incubation of cultures rather than due to any tolerance of the algal cells to the same concentration of active NTG.

In another experiment the survival of NTG-treated cells in basal NTG-free medium was studied. Log-phase cells were harvested by millipore filtration, washed with tris-maleic buffer (pH 8) and suspended in tris-maleic buffer (pH 6.5). This suspension was then divided into two aliquots. Into one aliquot NTG was added so as to obtain a final concentration of approximately 250 μg/ml. Both the reaction mixture and the control aliquot were continuously stirred on magnetic stirrers. Two-ml aliquots were withdrawn 30 and 60 min after the addition of NTG and after washing, the cells were suspended in 10 ml of fresh sterile ASM-1. From this suspension 0.05 ml aliquots (ca. 10^7 cells) or 0.5 ml aliquots were inoculated into flasks each containing 50 ml of ASM-1 medium. Cultures were incubated at 23—25C, 9,500 lux, with shaking. The results obtained are given in Fig. 2. The growth of controls (i.e., cells treated in buffer alone for 60 min) was better than that of treated cells

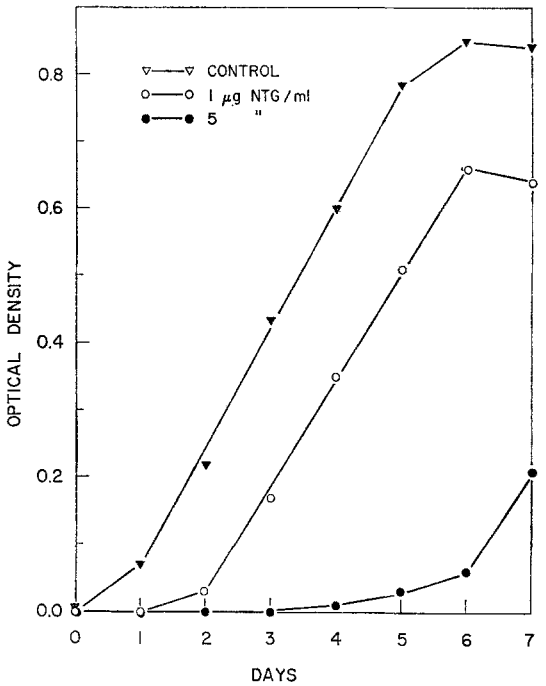

Fig. 1. Effect of NTG on growth of *A. nidulans*

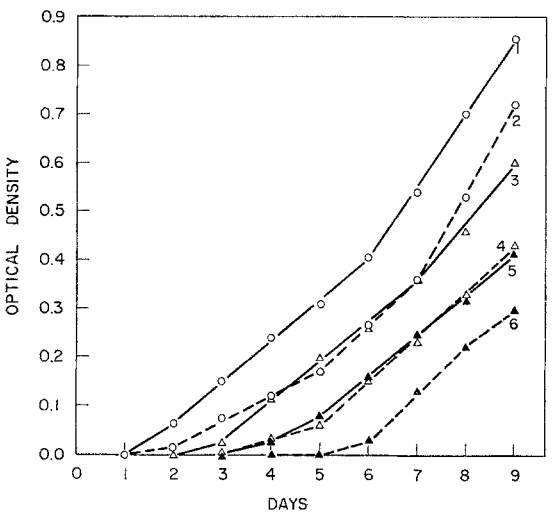

Fig. 2. Growth of NTG-treated *A. nidulans* in NTG-free basal medium curve *1*, untreated alga, inoculum 0.5 ml; curve *2*, untreated, inoculum 0.05 ml; curve *3*, NTG (30 min-treated), inoculum 0.5 ml; curve *4*, NTG-30, inoculum 0.05 ml; curve *5*, NTG-60, inoculum 0.05 ml

Correction added in print: In curve *5* inoculum should read 0.5 ml

both as regards the duration of the lag period and final optical density on the 9th day. The duration of the lag-phase in different cultures was found to be determined both by the duration of NTG treatment and by the inoculum concentration (Fig. 2).

Growth Following Successive Subcultures in NTG

The pattern of growth of the alga during successive subculture in culture media supplemented with different concentrations of NTG was studied with a view to determining whether or not the cells could acquire increased tolerance to NTG in this way. A second objective underlying the prolonged successive subcultures was to compare the mutagenic effects of a single NTG-treatment with those of repeated and prolonged treatments.

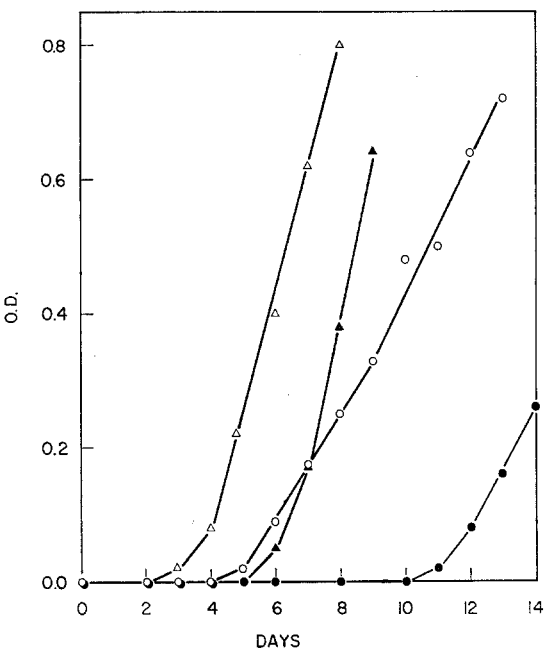

Fig. 3. Growth of *A. nidulans* in 5 μg NTG/ml (open symbols) and in 10 μg NTG/ml (solid symbols) during the 2nd and 3rd successive subcultures. Circles, second subculture; triangles, third subculture

When cells grown in 5 μg NTG/ml were inoculated into media containing 5, 10 or 20 μg NTG/ml, growth occurred in 5 and 10 μg/ml but not in 20 μg/ml. The lags in these concentrations were 5 days and 11 days respectively (Fig. 3). Cells from 10 μg NTG/ml of the second successive subculture were then inoculated into flaks containing 5, 10, and 20 μg NTG/ml. While no growth occurred in 20 μg NTG/ml, the lag-phase in

10 μg/ml was now reduced to 6 days. A similar procedure was repeated during subsequent subcultures. After five successive transfers in 10 μg NTG/ml, the alga was able to grow in 20 μg NTG/ml after lag periods varying between 16—12 days during the 6th to 10th successive subcultures.

Mutation Induction

One-week old cells of *A. nidulans* were treated with NTG in tris-maleic buffer at pH 5 (NTG is chemically stable at this pH) according to the method already described. Agar plates to be used for scoring streptomycin-resistant mutants were inoculated with $11-12\times10^7$ cells/plate whereas those for ultraviolet-resistant mutants were inoculated with $4-5\times10^7$ cells/plate. On the 10th day, after good confluent growth had occurred on all plates, the plates were either exposed to ultraviolet or overlaid with agar containing streptomycin. The ultraviolet-irradiated plates were stored in the dark for 16 h to avoid photoreactivation. After a 10-day incubation period the number of surviving colonies on each plate was counted. Mutants were scored both in untreated suspensions and in populations that had been treated with NTG either once or 10 times successively and the results obtained are given in the table.

Table. *Mutant frequencies of untreated* A. nidulans *and after single or 10 successive treatments with NTG*

Plate No.	Spontaneous			After single treatment			After 10 successive treatments	
	Sm^r-10 (10 μg/ml)	Sm^r-50 (50 μg/ml)	UV^r-15 (15 min)	Sm^r-10	Sm^r-50	UV^r-15	Sm^r-50	UV^r-15
1	3	1	31	81	46	72	0	32
2	2	3	84	115	138	9	0	49
3	1	2	0	102	5	95	0	21
4	5	4	52	36	82	63	0	84
5	3	1	5	16	17	16	2	60
6	8	2	3	63	60	27	0	57
Average/ plate	4	2	29	69	58	47	0	50

It can be seen that single treatments of *A. nidulans* with NTG result in an effective increase in the frequency of streptomycin-resistant mutants. The frequency of ultraviolet-resistant mutants is also increased by a factor of up to 2, after single or 10 successive subcultures in the presence of NTG.

Some attempts were made to find mutant clones resistant to 3-amino-1,2,4-triazole (Mann Research Laboratories). Agar plates inoculated with untreated or NTG-treated cells were incubated 10 days and then overlaid with agar containing 100 μg aminotriazole/ml. The bleaching as well as herbicidal and toxic effects of aminotriazole are well-known (AARONSON and SCHER, 1960); however, in this study it failed to effectively kill or bleach the background sensitive population and hence no discrete surviving mutants could be detected. A similar effect was also observed when plates were overlaid with agar containing penicillin (10—50 μg/ml) or NTG (30—40 μg/ml). However, NTG did exhibit a slow and weak bleaching effect and discrete NTG-resistant clones (0—17 per plate) became conspicuous after about 6 weeks of overlaying.

Attempts to select NTG-resistant mutants in liquid culture were unsuccessful. NTG (30 mg) was added into 200 ml culture suspension (in ASM-1 medium) and the culture incubated in dim light (about 1,000 lux) for 2 days after which it was incubated under normal conditions. The culture bleached within 10 days but did not revive even after a 6-week further incubation.

Discussion

In this study clear-cut results demonstrating the mutagenicity of NTG in *Anacystis nidulans* have been obtained with respect to the streptomycin-resistance character and single treatments of cells with NTG can lead to as much as a 15-fold increase in the frequency of streptomycin-resistant mutants over the spontaneous frequency. The mutagenic efficiency of NTG in inducing ultraviolet-resistant mutants, on the other hand, appears much less marked since the increase in mutant frequency in this case was less than two-fold. The spontaneous frequency of ultraviolet-resistant clones observed in this work (namely, an average of 29 colonies/plate) itself is rather high and might be attributed to holding the cells in the tris-maleic buffer for 60 min. Further attempts to check this effect are, however, necessary and are now in progress. In previous work (KUMAR, 1968a, 1968b) in which cells were not held in tris-maleic buffer, the mean frequency of spontaneous ultraviolet-resistant mutants was less than 5 colonies/plate.

Recent researches have indicated that bacterial resistance to streptomycin is bound to the function of the ribosome and in particular to its 30-S subunit (Cox *et al.*, 1964). In a sensitive organism, streptomycin treatment leads to misreading of the genetic code by misshaping the sensitive ribosome with the consequence that the organism synthesizes abnormal proteins and dies. In resistant cells, on the other hand, the site of attachment of streptomycin is altered so that either the streptomycin-

ribosome complex is not able to influence the geometry of the decoding site or the affinity for the antibiotic is decreased. The ribosomes have also been identified as the targets for the principal lethal action of NTG in *Escherichia coli* (CERDA-OLMEDO and HANAWALT, 1967). While the molecular mechanism of mutagenic action of NTG is not clearly understood, it would not be illogical to suppose that this might well be due to the effects of NTG on the enzymes related to nucleic acid replication. The recently reported isolation of RNA-polymerase from *Anacystis nidulans* (CAPESIUS and RICHTER, 1967) supports this view; to wit, NTG interaction with RNA-polymerase (or DNA-polymerase) may cause the enzyme to act as a phenotypically mutagenic polymerase.

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