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Canavanine-lnduced inhibition of growth and heterocyst differentiation in *Anabaena doliolum* and isolation of a canavanine-resistant mutant

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Abstract. The effect of the arginine analogue, canavanine on growth and heterocyst differentiation in the nitrogen–fixing alga *Anabaena doliolum* has been studied. The analogue inhibited growth and heterocyst differentiation at a concentration as low as 1 μM. The treated algal cells lacked conspicuous granular inclusions, whereas treatment with chloramphenicol led to increased synthesis of granules (probably cyanophycin granules). Exogenously added arginine completely reversed the effect of the analogue but lysine could only partially relieve the effect. A time course study with canavanine indicated inhibition of fresh protein(s) synthesis at all steps where a new class of proteins is synthesized so that the action of the analogue does not seem to be specific for a particular kind of protein. A mutant resistant to this analogue has been successfully isolated indicating that this alga does not show mutational immunity at least to the amino acid analogues unlike in the observation with different antibiotics. Our observations indicate that canavanine either directly inhibits protein synthesis or forms defective protein(s) which produces all the observed effects.

Keywords. Canavanine; inhibition of growth; protein synthesis; heterocyst; differentiation; *Anabaena doliolum.*

Introduction

Several filamentous nitrogen-fixing cyanobacteria show a characteristic pattern of heterocysts oriented in a one-dimensional array. One way of investigating the formation of such a spaced pattern in an organism is to alter the pattern with certain chemicals. Some amino acid analogues such as 7-azatryptophan, L-methionine-DL-sulfoximine and rifamycin (an inhibitor of DNA-dependent RNA polymerase) significantly change the heterocyst pattern (Mitchison and Wilcox, 1973; Wolk, 1975; Singh *et al.,* 1977; Agrawal and Kumar, 1978; Ladha and Kumar, 1978). A logical assumption is that the production of some compound, possibly an inducer of heterocyst development, is blocked by these chemicals or by their metabolic products (Mitchison and Wilcox, 1973). However, very few chemicals are known to act in the reverse way (*i.e*., blockage of the formation of an inducer of heterocyst development), except for a few specific protein synthesis inhibitors *e.g.*, chloramphenicol, actinomycin and combined nitrogen sources (the physiological inhibitor) which completely block heterocyst formation. In our study on amino

acid analogues, we have found that canavanine (2-amino-4-guanidino-oxy-butanic acid $NH_2C(=NH)$-NH-O-CH_2-$CH_3CH(NH_2)$) an analogue of arginine, strongly inhibits growth and heterocyst differentiation in a blue-green alga *Anabaena doliolum*. Canavanine causes an exponential inactivation of the bacterium *Escherichia coli* under conditions of low intracellular arginine (Schachtele and Rogers, 1965). Since the growth inhibition by canavanine is often reversed by arginine (Rosenthal, 1977), it has been suggested that canavanine interferes with arginine biosynthesis and/or utilization. Arginine plays an important role in the synthesis of cyanophycin granules (Simon, 1973, 1976) and the polar nodules of heterocysts in cyanobacteria and it was considered worthwhile to study the effect of canavanine on the various processes as no earlier study had been made. In this paper we describe the effect of canavanine on growth and heterocyst differentiation and the reversal of its toxicity by arginine and lysine, and compare these with the effects of a few other analogues.

Materials and methods

Organism and culture conditions

Anabaena doliolum was isolated from the local rice fields. The alga was made unialgal and axenic adopting Standard microbiological techniques and was routinely grown in Allen and Arnon's (1955) medium with or without combined nitrogen (10 mM KNO_3 and/or 2 mM NH_4C1) at 27±2°C in a culture room. As this alga sporulated *en masse* at the end of the stationary phase of growth, a clonal population was very easily raised from a single spore. The cultures were illuminated with cool white fluorescent tube lights at 2000 lux for 16 h daily. All experiments were performed with the cultures obtained at the log phase (6-day old). Growth experiments were carried out in culture tubes by measuring the absorbance in a Bausch and Lomb Spectronic 20 colorimeter at 665 nm. Heterocyst frequency was determined as % of total cells by counting at least 10 filaments (*ca* 600-1000 cells). Cyanophycin granules were observed microscopically and compared with those in untreated controls. The cytochemical test for cyanophycin granules was made by the method of Fuh (1968). Filaments were stained in a saturated solution of carmine in 45% acetic acid for 10-15 minutes. The cyanophycin granules developed a distinct red colour after staining. However, the presence of other granules was also taken into account. To obtain non-heterocystous cultures, the cultures were previously grown in KNO_3 (0.02 M).

Isolation of canavanine-resistant mutant

Spores of *Anabaena doliolum* were collected by centrifugation at 3000 *g* for 10 min, washed at least 3-4 times and then incubated in 10 ml (Allen and Arnon's KNO_3) medium for 48 h. This resulted in swelling of about 90% of the spores and the colour also became more deeply bluish-green. From this stage of spore suspensions, aliquots were withdrawn and treated with N-methyl-N'nitro-N-nitrosoguanidine (methylnitrosoguanidine) solution (100 μg/ml at pH 7) for 30 minutes (this treatment permits about 50% survival). After 3-4 washings and centrifugations of the methylnitrosoguanidine-treated spore suspensions, 10^6 to 5×10^7 colony forming

units were plated on *ca* 30 agar plates each having l0µg/ml canavanine. Then, the plates were transferred to the culture room and probable resistant clones arising after two weeks were picked up and tested for growth in canavanine supplemented plates as well as in a liquid culture medium. After 3-4 subcultures stable resistant clones were isolated and maintained in canavanine supplemented slants.

Chemicals

L-Canavanine, L-arginine, L-lysine, L-norleucine. α-methyl-DL-methionine, N-formyl-DL-methionine, α-methyl-DL-aspartic acid, L-methionine-DL-sulfoximine, DL-7-azatryptophan and chloramphenicol were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Methylnitrosoguanidine was kindly provided to us by Dr. C. Van Baalen of Marine Science Institute, USA, University of Texas. Other chemicals used were of the highest purity available from British Drug House (Glaxo), New Delhi. Solutions of all amino acids, amino acid analogues, chloramphenicol and methylnitrosoguanidine were sterilized by membrane filtration.

Results

Effect of canavanine on growth

Figure 1 shows that the addition of canavanine at a concentration as low as 1 µM results in inhibition of the algal growth. No growth occurred at concentrations of 10 µM or 100 µM of the chemical. Growth was not resumed even 12 days after the treatment. The transfer of the filaments treated for 48 h with 10 µM canavanine in

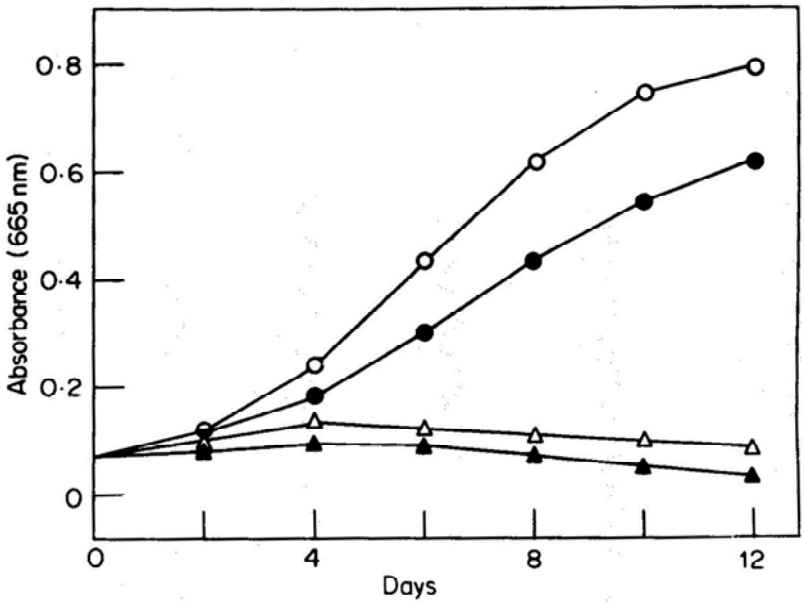

Figure 1. Effect of different concentrations of Lrcanavanine on growth of *Anabaena doliolum* in medium free of combined nitrogen. Control, (○); Canavanine, 1 µM (●); 10 µM (△); and 100 µM (▲).

an analogue-free medium also failed to elicit resumption of growth (data not given). Our results on growth inhibitions are in agreement with similar observations on *E coli* and *Chlamydomonas reinhardii* (Schachtele and Rogers, 1965; McMahon, 1971). Microscopic examination of the canavanine-treated alga showed complete loss of the characteristic granular inclusions, while such granules became more frequent following treatment of the alga with chloramphenicol. This observation is based on the presence or loss of characteristic red coloured granular inclusions following acetocarmine staining of the filaments. Most probably, these granules represent the cyanophycin granules comprising of aspartic acid-arginine co-polymers.

Reversal of canavanine toxicity by arginine and lysine

The effect of canavanine (10 μM) is reversed by the simultaneous addition of 100 μM arginine (figure 2). There was no loss of granules from the stationary phase cultures as distinct red granules were observed in all the filaments of the culture after acetocarmine staining. Arginine alone stimulated growth and also significantly repressed heterocyst frequency (figure 2 and table 1). There is no report of an arginine-induced stimulation of growth except in *Plectonema boryanum* where arginine inhibited nitrogenase activity by acting as a nitrogen source (Nagatani and Haselkorn, 1978). Lysine did not relieve the canavanine-induced inhibition of growth as efficiently as arginine (figure 2). There was very poor growth with lysine + canavanine and this finding was in contrast to an earlier study made on fungi, where lysine completely reversed inhibition of growth by canavanine (Walker, 1955). Our finding is consistent with that of Aslam *et al.* (1978), where arginine proved more effective than lysine in reversing canavanine-induced inhibition of the induction of nitrate reductase in corn roots. Lysine alone did not show a significant effect either on growth or heterocyst frequency.

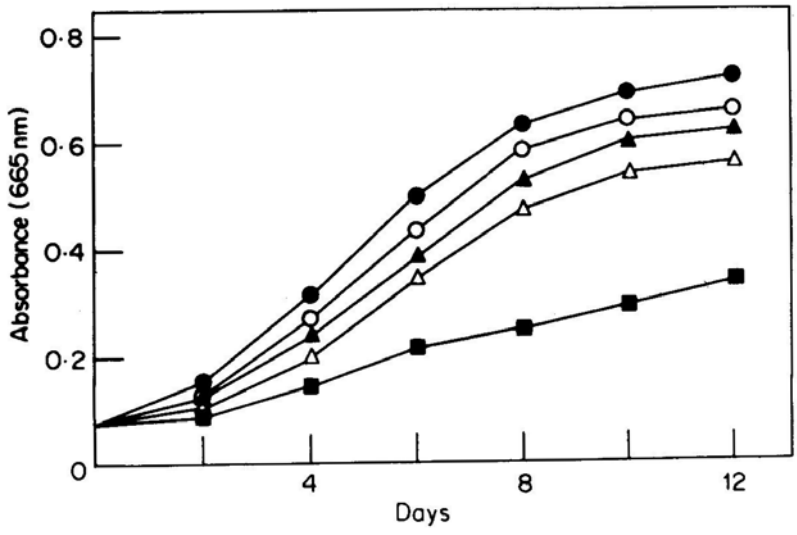

Figure 2. The effect of arginine and lysine on growth and on the reversal of L–canavanine induced toxicity. Control, (△); arginine 100 μM, (●); lysine 100 μM (▲); arginine 100 μM + canavanine 10 μM, (○); and lysine 100 μM + canavanine 10 μM, (■).

Table 1. Heterocyst frequency[a] in different concentrations of L–canavanine, arginine and lysine in a culture medium free-of organic nitrogen compounds.

Addition (μM)	Proheterocysts %	Mature heterocysts %
None	2	4.5
L-canavanine (1.0)	0	0
L-canavanine (10.0)	0	0
L-lysine (100)	1.5	4.0
L-arginine (100)	1.5	2.5
L-arginine (100) + L-canavanine (10)	1.5	4.5
L-lysine (100) + L-canavanine (10)	1.5	3.0

[a] Heterocyst frequency was counted after 72 h, heterocysts having thick wall and developed polar nodules being counted as mature heterocysts.

Effect of canavanine on heterocyst production

Canavanine (1 μM or above) completely inhibited heterocyst formation (table 1). The simultaneous addition of arginine (100 μM) relieved the inhibition, and permitted the filaments to differentiate into heterocysts with a frequency, comparable to that observed in the controls. However, there was only 3% hetorocyst formation in a lysine + canavanine containing medium. This is consistent with the noted partial recovery of canavanine inhibited growth.

Effects of some other amino acid analogues on heterocyst differentiation

Amino acid analogues have been categorised into three groups *viz.,* group a, b and c on the basis of their effect on growth and heterocyst formation. Amino acid analogues of group (a) inhibited growth but had no effect on heterocyst frequency. In group (b), inhibition of growth was accompanied by a drastic change in hetero cyst frequency and spacing. L-norleucine, α-methy 1-DL-methionine, N-formyl DL-methionine and α-methyl-DL-aspartic acid belong to this group. With 1 μM methionine sulphoximine, the heterocyst frequency increased to 8-9%, whereas with 100 μM 7-azatryptophan, the frequency was 13-14%. Finally the only compound in group (c), canavanine, inhibited growth as well as heterocyst formation. As shown earlier, 1 μM or higher concentrations of canavanine completely inhibited heterocyst formation. This shows that these effects of different amino acid analogues are similar.

Time-course study with canavanine and chloramphenicol on heterocyst differen-Tiation

The effect of canavanine was compared with that of the known protein synthesis inhibitor, chloramphenicol in a short term experiment on heterocyst differentiation. The concentrations:of canavanine (1. μM) and chic ramphenicol (25 μg/ml) chosen

were those which had been found to be nontoxic as judged by algal viability and survival after treatment with these concentrations and following the transfer of treated cultures into drug-free medium. Canavanine or chloramphenicol was added to non-heterocystous, nitrate-grown filaments at 0, 4, 8, 12 and 24 h after incubation of these filaments in nitrogen-free medium.The addition of either of the chemicals to cultures in a heterocyst-inducing medium at 0, 4, and 8 h caused complete suppression of heterocyst differentiation. Heterocyst formation was not suppressed when the chemicals were added at 12 or 24 h. This observation showed that canavanine blocked the formation of proteins, some of which might be required for heterocyst differentiation and which may be formed within 8 h following transfer of a non-heterocystous culture to a heterocyst induction medium.

Canavanine -resistant mutant

The canavanine resistance mutation frequency following mutagenesis using nitrosoguanidine was much higher than the frequency of spontaneous mutation (table 2). The canavanine-resistant mutant strain showed a slightly faster growth rate than the parent strain. The heterocyst frequency also increased from 4.5 to 5.5% in the mutant. However, the filaments were highly granulated in contrast to the loss of granules in the canavanine-treated wild type. Further the mutant did not require arginine for normal growth, but showed better growth with canavanine than without it. The mutant alga showed resistance to canavanine even after its growth in a canavanine-free medium for a long time.

Table 2. Mutation frequency, generation time[a] and heterocyst frequency of parent and Cnv^R (L-canavanine resistant mutant)

Strain and growth condition	Mutation frequency		Generation time (h)	Heterocyst frequency (%)
	spontaneous	Induced (by NTG)		
Parent (combined nitrogen-free medium)	–	–	50	4.5
Cnv^R (with L-canavanine, 10 ug/ml)	1.52×10^{-7}	5.63×10^{-6}	46	5.5
Cnv^R (Without L-canavanine)	–	–	52	5.0

[a] Values given are average of triplicates and represent the time taken by the growing algal culture to double its original absorbance in the exponential phase of growth; time was deter mined directly from the growth curve.

1 μM canavamne = 0.17 μg/ml.

Discussion

The above data indicates the following tentative conclusions concerning the effect of canavanine.

Firstly, the action of canavanine is quite different from that of the other amino acid analogues discussed above. It was highly toxic even at very low concentrations. The observed reversal of toxicity by the simultaneous addition of arginine favoured the competitive utilization of canavanine by the alga. Arginine was more effective than lysine in reversing the canavanine-induced inhibition of growth and heterocyst differentiation. This is consistent with earlier observations of the effects of canavanine on the growth of maize embryos and on the induction of nitrate reductase in corn roots (Rosenthal, 1977; Aslam *et al.,* 1978). The paucity of cyanophycin granules in canavanine-treated material supports the findings of Simon (1976), who showed that the incorporation of (^{3}H)-labelled arginine by multi L-arginyl-(poly) aspartic acid synthetase was insensitive to the addition of a variety of antibiotics including chloramphenicol, erythromycin, streptomycin, tetracycline, rifamycin, 7-methyltryoptophan and 7-azatryptophan, However, the addition of canavanine inhibited (upto 72%) the incorporation of ($_{3}$H)-arginine and the inhibition could be overcomeby increasing the concentration of L-arginine in the reaction mixture. Chloramphenicol-induced formation of cyanophycin granules in this alga confirms the earlier reports in *Agmenelum quadruplicatum* and *Anabaena cylindrica* (Ingram *et al,* 1972; Simon, 1973).

Secondly, the inhibition of heterocyst differentiation by canavanine is unique in the context of the known effects of other amino acid analogues. In the case of L-methionine-DL-sulfoximine it is known that glutamine synthetase is inhibited and it leads to a decrease in the intracellular level of glutamine and excretion of ammonia into the medium resulting in changes in the frequency and spacing of heterocysts (Stewart and Rowell, 1975; Ownby, 1977; Singh *et al.,* 1977; Ladha and Kumar, 1978). On the other hand 7-azatryptophan was incorporated, in the place of tryptophan resulting in the formation of defective proteins. Mitchison and Wilcox (1973) proposed that this defective protein was solely responsible for the observed alteration in heterocyst pattern formation. No previous report on the influence of canavanine on the heterocysts is available. However, the action of canavanine as an arginine analogue has been summarized in the recent reviews (Norris and Lea, 1976; Rosenthal, 1977). It has been well established that heterocyst differentiation is dependent on the synthesis of a nascent protein and also on a product of the photosynthetic process (Singh and Srivastava, 1968; Singh and Kumar, 1971; Tyagi, 1975; Haselkorn, 1978). Chemicals affecting either of the two processes inhibited heterocyst differentiation (Tyagi, 1975). From our study, the effect of canavanine appeared to be primarily on the synthesis of proteins. Possibly, canavanine inhibited protein synthesis by acting as a substrate for arginyl-tRNA. The inhibition of protein synthesis in a heterocyst-inducing medium by canavanine resulted in the inhibition of heterocyst differentiation. In this case preformed cellular proteins may be insufficient to fulfil the complete manifestation of heterocyst differentiation. This inhibition of protein synthesis was not stage-specific.

This is indicated by the data of the time-course experiment where even after 12 h (the time for proheterocyst formation), the maturation of heterocysts was blocked. However, in addition to the direct inhibition of nascent protein synthesis, canavanine might also be directly incorporated into polypeptides resulting in the formation of a defective protein(s). Such defective proteins could be the cause of inhibition of growth and also heterocyst differentiation. Formation of defective canavanyl protein(s) has been shown in *E. coli* by Sachachtele *et al.* (1968).

The mechanism of canavanine resistance in the mutant strain is not known. A possible cause of resistance might be a defective arginyl-tRNA synthetase in the mutant. Similar mutants resistant to canavanine were previously isolated in bacteria and *Chlamydomonas* (Sachachtele *et al.,* 1968; McMahon, 1971).

The mutant is interesting in view of the generally observed mutational immunity of *A. doliolum* to certain resistance markers, e.g., antibiotic resistance (Ladha and Kumar, 1978). There is only one previous report of an ethionine-resistant mutant of a non-nitrogen fixing alga *Plectonema boryanum* (Hentschel *et al.,* 1978) and hence the isolation of this mutant should greatly advance our understanding of biosynthesis and regulation of amino acids. This mutant was stable even after several subcultures and canavanine-free medium and thus seems to offer a suitable genetic marker in the absence of other antibiotic markers in this alga.

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