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Simultaneous Utilization of Urea and Nitrate by the Cyanobacterium *Anabaena doliolum*

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(i) Urea and nitrate were utilized simultaneously when both the nitrogen sources are simultaneously available in the growth medium; (ii) uptake of urea and nitrate occured at different sites and its repression depends on ammonium assimilation product (feed-back system); (iii) the competitive interaction between urea and nitrate utilization did not take place at the level of uptake during short-term experiments; and (iv) urea and nitrate uptakes were not linked with the ammonium assimilating enzyme, glutamine synthetase during short-term experiments.

Key words: ***Anabaena doliolum*, Cyanobacterium, Nitrate uptake, Urea uptake**

Introduction

Cyanobacteria are oxygenic, photosynthetic prokaryotes which have long been recognized as having potential in biotechnology. Nitrate and ammonium are the main combined nitrogen sources for the growth of this group of organisms under natural conditions and have received more attention than organic nitrogen. Urea can also support excellent growth of some cyanobacteria (Neilson & Larsson 1980, Singh 1985, 1991c, Singh & Rai 1989, 1990) but little is kown about the mechanism(s) responsible for its uptake and metabolism (Healey 1977, Carvajal et al. 1982, Rai & Singh 1987a,b, Singh 1988, 1990a,b, Singh 1991a,b,c, Singh 1992a, Singh & Ahmad 1989, Singh & Rai 1990 Ge et al 1990) and further studies are however, required to understand the mechanism(s) of urea uptake and utilization in other cyanobacteria.

Nitrate assimilation is repressed when adequate level of the end product ammonium is present in the immediate environment (Rai & Singh 1982), whereas urea assimilation does not show any repression in the presence of ammonium (Singh 1988, 1992b). When nitrate and ammonium both are available in the growth medium, a preferential utilization of ammonium over nitrate takes place (Rai & Rai 1977, Rai & Singh 1982) whereas urea is taken up preferentially when urea and ammonium are simultaneously available to *Nostoc muscorum* cells (Singh 1992b). In contrast, report is also available on the simultaneous utilization of nitrate and ammonium (Rai & Rai 1977) and urea and ammonium (Healey 1977, Singh 1992b). However, nothing is known about the interaction between urea and nitrate during uptake. It is also not yet clear whether or not urea and nitrate are utilized simultaneously by the cyanobacterial cells.

Now a consensus is evolving that recognises the importance of uptake in the substrate utiliza-

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tion. In cyanobacteria such as *Anabaena doliolum*, there are no vacuoles to store urea or nitrate in the usual way. Thus, hydrolysis or reduction of urea or nitrate immediately follows their uptake making a distinction of these two processes difficult. The separation of urea or nitrate uptake from their subsequent hydrolysis or reduction may be helpful in obtaining information regarding the regulation of the uptake process.

The present paper reports that *Anabaena doliolum* utilizes both urea and nitrate simultaneously when the nitrogen sources are available in the growth medium at the same time and there is no competitive interaction between urea and nitrate at the level of uptake during short-term experiments. Furthermore, it is also shown that short-term uptake of urea and nitrate (upto 1 hr) is independent of glutamine synthetase acitvity.

Materials and Methods

Organisms and Culture Conditions

Anabaena doliolum Bharadwaja was axenically grown in modified Chu No. 10 medium (Safferman & Morris 1964) without any combined inorganic nitrogen source (N_2 -medium) at 24±1°C and illuminated with day-light flourescent tubes (intensity 15 W m^{-2} on the surface of the vessels) for 14 hr day^{-1}. ATP (50 μM), L-methionine-DL-sulphoximine (MSX, 250 μM) and ammonium chloride (500 μM) were added to the cultures when required. These cultures were incubated for 2 hr under normal growth conditions so as to allow the entry of the chemicals into the cells. The *p*H of the medium was adjusted to 7.3 in all the cases.

Uptake Assay

Uptake of urea or nitrate was assayed by measuring the depletion of these ions from the external medium as described before (Singh 1988, Rai & Singh 1982). Exponentially growing cells were harvested by centrifugation (5000 g, 10 min), washed twice with sterile distilled water and resuspended in sterile N_2-medium. Uptake was initiated by the addition of urea (2 mM) and calcium nitrate (1 mM). Unless otherwise stated, the uptake experiments were conducted at a light intensity (from day- light fluorescent tubes) of 15 W m^{-2} at 24±1°C. From the samples withdrawn at regular intervals (30 min), the cells were removed by rapid centrifugation and the cell-free supernatants were analysed for residual urea or nitrate. The uptake rates were estimated from linear portion of the curves (uptake during 0-30 min).

Analytical Methods

Urea was determined in the supernatant fluid using diacetylmonoxime (Sigma Technical Bulletin No. 535, 1980) and nitrate was estimated by using brucine-H_2SO_4 (Nicholas 1953). Cellular protein was estimated by the method of Lowry et al. (1951) standardized with bovine serum albumin.

Results and Discussion

The objective of this study was to investigate whether urea and nitrate were simultaneously utilized by *A. doliolum* cells or not when both the nitrogen sources were available in the growth medium. In consequence the uptake of urea and nitrate were measured over a period of 1 hr by incubating the *A. doliolum* cells in N_2 -medium supplemented with 2 mM urea and 1 mM calcium nitrate. This was performed in view of the facts that urea and nitrate uptake during 1 hr is unaffected by its subsequent hydrolysis and reduction (Singh 1988, Rai & Singh 1982). The data in table 1 show that the *A. doliolum* cells incubated in the presence of urea and nitrate, utilized both the nitrogen sources simultaneously at similar rate as observed with the control cells incubated with only one nitrogen source. Thus, it may be assumed that the uptake of urea and nitrate occurs simultaneously at different uptake sites and there is no competitive interaction between both the nitrogen sources at the level of uptake during short-term experiments.

Ammonium ion severely inhibited the nitrate uptake whereas it did not inhibit the urea uptake at all (table 1, urea + NH_4Cl; NO^-_3 + NH_4Cl). Thus, it seems plausible that two uptake systems also participate in the uptake of urea and ammonium in *A. doliolum* like *N. muscorum* (Singh 1992b). In cyanobacteria, the primary ammonia assimilating enzymes are glutamine synthetase (L-glutamine : ammonia ligase, ADP forming EC 6.3.1.2) in conjugation with glutamate synthase (L-glutamine :2-oxoglutarate amino transferase, EC 1.4.7.1) (Meeks et al. 1977, 1978). MSX, a glutamate analogue induces a drastic irreversible inhibition of glutamine synthetase (GS), thus preventing the assimilation of ammonium by this route (Stewart & Rowell 1975). MSX did not inhibit the urea and nitrate uptake; however, it prevented the ammonium inhibition of nitrate uptake (table 1, urea + MSX; NO^-_3 + MSX; NO^-_3 + NH_4 Cl + MSX). Thus, in analogy to what happens with nitrate uptake in *N. muscorum* (Rai & Singh 1982) ammonium must be assimilated via GS in order to inhibit nitrate uptake. Thus, it may be concluded that short-term uptakes of urea and nitrate are not linked with the ammonium assimilating enzyme, GS. Furthermore, the cells treated with MSX, not only showed the recovery in nitrate uptake from ammonium inhibition but also exhibited an increased rate of nitrate uptake (table 1: urea + NO^-_3 + NH_4Cl + MSX). Thus, the regulatory system modulating nitrate uptake via the active system seems to involve products of the ammonium assimilation via GS. Similar results on MSX interaction with nitrite uptake have also been reported in *Nostoc* ANTH (Singh 1992c).

The cells receiving ATP exogenously showed an increased rate of urea and nitrate uptake to that of control cells (without ATP) (table 1, urea + ATP; NO^-_3 + ATP). In our previous papers (Rai & Singh 1982, Singh 1988, Singh & Ahmad 1989) we have reported that nitrate and urea uptakes are active processes linked to the proton gradient which is formed either by photophosphorylation or ATP hydrolysis. Thus, it is likely that the competition between nitrogen sources may lie at ATP level if the uptake processes are energy-dependent.

Table 1 *Utilization of urea and nitrate by the cyanobacterium* ***Anabaena doliolum*** *in the presence and absence of ammonium chloride, ATP and MSX*

Addition or incubation condition	Substrate uptake (nmol (μg protein)$^{-1}$ hr^{-1})	
	Urea	Nitrate
Urea	556.5	-
Urea + MSX	567.9	-
Urea + ATP	637.0	-
NO^-_3	-	109.30
NO^-_3 + MSX	-	135.20
NO^-_3 + ATP	-	195.32
Urea + NO^-_3	550.0	107.99
Urea + NO^-_3 + NH_4 Cl	549.5	4.97
Urea + NO^-_3 + NH_4 Cl + MSX	562.5	129.50
Urea + NO^-_3 + NH_4 Cl + ATP	627.3	99.27

A. doliolum cells grown in N_2 -medium were washed thoroughly with sterile distilled water and resuspended in the same medium supplemented with urea (2 mM) and calcium nitrate (1 mM). Urea and nitrate uptake were determined upto 1 hr as described in Materials and Methods. MSX (250 μM), ATP (50 μM) and ammonium chloride (500 μM) were added to the cultures 2 hr prior to the addition of urea and nitrate under normal growth conditions so as to allow the entry of these chemicals into the cells.

Since, nitrate and ammonium are also utilized simultaneously (Rai & Rai 1977) and both the uptake processes are also energy- dependent (Rai & Singh 1982, Rai et al. 1984), the competition between nitrate and ammonium may also take place at ATP level. Further to establish whether ammonium inhibition of nitrate uptake lies at ATP level, the uptake of nitrate was studied in the presence of ammonium and externally added ATP (table 1, Urea + NO^-_3 + NH_4 Cl + ATP). The cells treated with ATP and NH_4 Cl showed the recovery in nitrate uptake from ammonium inhi-

bition, indicating that if the supply of ATP is sufficient to meet the requirement of nitrate uptake, nitrate may be taken up even in the presence of ammonium. Thus, the competition between nitrate and ammonium utilization also occurs at ATP level when both the nitrogen sources are simultaneously available during short-term experiments.

It is proposed that (i) urea and nitrate are utilized simultaneously by *A. doliolum* cells when both the nitrogen sources were simultaneously available in the growth medium; (ii) uptake of urea and nitrate occurs at different sites and its repression depends on the ammonium assimilation product (feed-back system); (iii) the competitive interaction between urea and nitrate utilization does not take place at the level of uptake during short-term experiments; and (iv) during short-term experiments, urea and nitrate uptakes are not linked with the ammonium assimilating enzyme, GS.

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