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Fructose utilization by the cyanobacterium *Anabaena variabilis* studied using whole filaments and isolated heterocysts

Bent Borg Jensen*

Institute of Biochemistry, Odense University, Campusvej 55, DK-5230 Odense M, Denmark

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Abstract. We have investigated the utilization of [^{14}C]-fructose by whole filaments and isolated heterocysts of *Anabaena variabilis* ATCC 29413, a strain which is capable of fructose-dependent heterotrophic growth. The experimental conditions were chosen such that both transport and subsequent metabolism were studied. The apparent K_m for fructose was 60 μM, close to the results of previous studies. Rates of fructose utilization were the same in light and darkness. When photosynthetic CO_2 fixation was possible, almost all the label appeared as cell-carbon. In darkness or in the presence of DCMU appreciable amounts of label were released as CO_2. Isolated heterocysts with high rates of endogenous metabolism were not capable of utilizing added fructose at significant rates. The effects of oxygen concentration on the metabolism of added fructose in darkness showed that uptake was saturated at low pO_2 values. Increasing the pO_2 values lead to an increase in the ratio between the lable released as CO_2 and that recovered as cell-carbon. These results suggest that fructose is taken up only by the vegetative cells but carbon derived from added fructose can be released as CO_2 as a result of respiration in the heterocysts. Fructose utilization was inhibited by uncouplers. The greatest inhibition was found when both (delta) (psi) and (delta) pH were abolished. High concentrations of erythrose inhibited fructose utilization. None of the other potential analogs tested had any effect.

Key words: *Anabaena variabilis* — Heterocysts — Fructose utilization — O_2-affinities — pmf

When grown without combined nitrogen, many filamentous cyanobacteria show a differentiation into two distinct cell types: vegetative cells and heterocysts (Fogg 1949; Rippka et al. 1979). Heterocysts are believed to provide the anaerobic environment necessary for operation of the oxygen-sensitive enzyme nitrogenase (Wolk 1982). It is generally believed that in vivo, heterocysts are metabolically dependent on vegetative cells. They lack a functional photosystem II and do not possess ribulose-1,5-bisphophate carboxylase and are therefore unable to fix CO_2 photosynthetically. They are presumably supplied with fixed carbon by the vegetative cells. Although a lot of effort has been put into attempts to determine which carbon compound or compounds are transported from the vegetative cells to the heterocysts, this compound still remains to be identified.

Photoautotrophic growth is the most common mode of growth of cyanobacteria. However, several cyanobacteria have been shown to be capable of heterotrophic growth in the dark at the expense of various sugars which act as both carbon and energy source. Studies on sugar uptake by cyanobacteria have mainly concentrated on the assimilation of glucose (Pelroy et al. 1972; Raboy et al. 1976; Raboy and Padan 1978; Beauclerk and Smith 1978; Smith 1983). These studies have indicated the presence on glucose assimilating strains of permeases showing K_m-values for glucose of about 0.1 – 1.0 mM (Raboy and Padan 1978; Beauclerk and Smith 1978; Smith 1982, 1983; Flores and Schmetterer 1986).

Fructose is a good substrate for growth for many cyanobacteria (Watanabe and Yamamoto 1967; Hoare et al. 1971; Wolk and Schaffer 1976; Haury and Spiller 1981; Jensen 1983; Flores and Wolk 1985). So far fructose uptake has only been studied in the two facultatively heterotrophic cyanobactria, *Anabaena variabilis* ATCC 29413 (Haury and Spiller 1981) and *Nostoc* sp. ATCC 29150 (Schmetterer and Flores 1988).

Fructose has been shown to influence the morphology and the metabolism of *A. variabilis* in several ways. Fructose added to cultures of *A. variabilis* produces marked morphological and ultrastructural changes in vegetative cells and heterocytes. As shown by Lang et al. (1987) both vegetative cells and heterocysts of *A. variabilis* be-

Present address and address for offprint requests: National Institute of Animal Science, Department of Animal Physiology and Biochemistry, Foulum Research Center, DK-8830 Tjele, Denmark

came larger, were filled with glycogen granules and had fewer thylakoid membranes when the cyanobacteria were grown in fructose-supplemented medium. Fructose has also been shown to produce marked changes in cell yields, protein content, and chlorophyll formation (Haury and Spiller 1981), to increase acetylene reduction (Haury and Spiller 1981; Jensen 1983; Jensen et al. 1986) and to double the heterocyst frequency (Haury and Spiller 1981).

We have previously shown (Jensen et al. 1986) that heterocysts isolated from fructose-grown filaments of *A. variabilis* had higher acetylene-reduction capacity than heterocysts isolated from photoautotrophically-grown filaments. In this paper we report the results of experiments designed to provide information about the factors influencing fructose uptake in whole filaments of *A. variabilis* and an investigation of the possibility of fructose uptake by isolated heterocysts prepared from fructose-grown cyanobacteria.

Materials and methods

Organism and growth conditions

Anabaena variabilis Kütz ATCC 29413 was cultivated in $BG11_0$ medium (Rippka et al. 1979) supplemented with 10 mM fructose. The cyanobacteria were grown at 30°C under continuous illumination (100 $\mu E/m^2/s$ of photosynthetically active radiation incident on the surface of the culture), either in 100 ml conical flasks containing 50 ml medium on a shaker or in 1.5 l air-sparged batches in 2 l fermenters with magnetic stirring. The cyanobacteria were harvested in the exponential growth phase and resuspended in growth medium without fructose. This medium was used for all measurements.

Fructose uptake measurements

[^{14}C]-fructose uptake by intact filaments was measured using freshly harvested filaments concentrated by centrifugation to 10 µg Chl/ml. The reaction volume was 1 ml. Assays were performed in 10 ml vials fitted with serum stoppers. The vials were modified by cementing a small cup made from a cut-off glass test tube to the inside wall. To absorb CO_2 a piece of filter paper moistened with 1 mM NaOH was placed in the cups and the cyanobacteria in the remaining portion of the vial. Unless otherwise stated the gas phase was air. Assays were initiated by addition of [U-^{14}C]-fructose (specific activity 6.7 µCi μmol^{-1}) and terminated by injection of 0.3 ml 25% trichloroacetic acid (TCA) into the vial through the stopper. The reaction time was 20 min and temperature 30°C. In experiments with analog and inhibitors, filaments were preincubated for 20 min with these compounds before initiating the experiment. After the termination of the reaction, 0.5 ml of the cell suspension was withdrawn and the filaments or heterocysts were collected on a 0.45 µm Millipore filter by filtration, washed with 10 ml cold growth medium and dried at room temperature. After drying the filters were immersed in a scintillation cocktail and counted using a scintillation counter. Radioactivity in samples of cell suspensions, filtrates and $^{14}CO_2$ absorbed in 1 M NaOH on filter paper was also measured by scintillation counting after addition to scintillation liquid.

Isolation of heterocysts

Heterocysts were isolated and assayed as described by Jensen et al. (1986) usig 200 mM sucrose in the isolation/assay medium (40 mM Hepes-KOH, pH 7.6, 1 mM $MgCl_2$). The lysozyme-treated cyanobacteria were disrupted by a single passage through a French pressure cell at 22.8 MPa. All buffers used were made anaerobic by sparging with argon or N_2 scrubbed free from O_2 by passing over hot (110°C) BASF catalyst R3-11.

Acetylene reduction

C_2H_2-reduction assays were performed in 10 ml vials fitted with rubber septa. The reaction volume was 1 ml, and the gas phase was 10% C_2H_2 in air. C_2H_2 was generated by addition of CaC_2 to H_2O in a Burris bottle (Burris 1974). Assays were initiated by the addition of filaments and terminated by the addition of 0.3 ml 25% TCA. Gas samples (0.5 ml) were removed with 1.0 ml plastic syringes and ethylene was measured on a PYE 104 gas chromatographic unit equipped with a flame ionisation detector using a column of Porapak N operated at 50°C with N_2 as carrier gas.

Chlorophyll a (Chl) determination

One-millilitre samples were extracted with 9 ml 100% methanol for 10 min. The concentration of chloropyll a (Chl) was determined from the absorbance of the centrifuged extract at 665 nm using an extinction coefficient of 74.5 l g^{-1} cm^{-1} (MacKinney 1941).

Results and discussion

[^{14}C]-fructose uptake by whole filaments

Since fructose is a metabolisable sugar, uptake of ^{14}C from labelled fructose depends on both transport and the subsequent metabolism and incorporation. Nevertheless uptake studies are useful in providing information about relative rates of overall uptake and incorporation, and patterns of preferential utilization or inhibition.

To ensure that the measurements were made under conditions where uptake of fructose was linear, time course studies were undertaken. Fructose uptake in the light was constant for at least 40 min in experiments carried out with an initial fructose concentration of 200 µM and a cyanobacterial density corresponding to 10 µg Chl/ml (results not shown).

Table 1 shows the distribution of radioactivity from [^{14}C]-fructose after incubation of whole filaments of *A. variabilis* either in light or darkness. Also shown are the results of an experiment in which TCA was added before initiating the incubation. In all three experiments the recovery was close to 100%. Almost no ^{14}C was recovered in the CO_2-fraction in the experiment carried out in the light. In the experiment carried out in the dark, on the other hand, about half of the ^{14}C taken up was recovered as CO_2. The total amount of ^{14}C taken up (^{14}C in cells + ^{14}C as CO_2) was about equal in light and darkness. Addition of TCA to the reaction mixture before incubation completely blocked fructose uptake. In the succeeding experiments, samples incubated in the presence of TCA were used as controls.

Figure 1 shows the rate of fructose uptake in the light by whole filaments as a function of the initial fructose concentration. The curve through the points corresponds to the calculated best fit to the Michaelis-Menten equa-

Table 1. Distribution of radioactivity from [^{14}C]-fructose incubated with intact filaments of *Anabaena variabilis*

Incubation conditions	^{14}C added (dpm)	^{14}C recovered				Recovery (%)
		in cells (dpm)	as CO_2 (dpm)	in SN[a] (dpm)	total (dpm)	
Dark	390000	48000	58000	271000	377000	97
Light	390000	100000	2000	282000	394000	101
Light + 5% TCA	390000	10000	44	370000	381000	98

[a] SN: supernatant

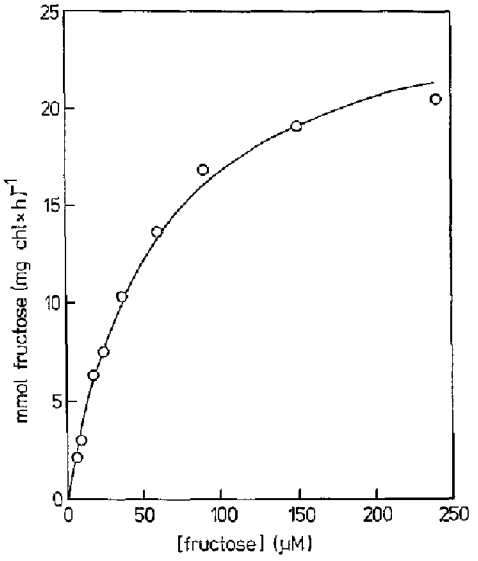

Fig. 1. Rate of fructose uptake in the light by whole filaments as a function of the initial fructose concentration. Freshly-harvested filaments were concentrated by centrifugation to 10 µg chl/ml in fresh growth medium without fructose. At time zero, ^{14}C-fructose was added to the concentrations shown, and the cyanobacteria were incubated at 30°C in the light. Reactions was terminated after 20 min by addition of TCA and uptake of [^{14}C]-fructose measured as described in the Methods section

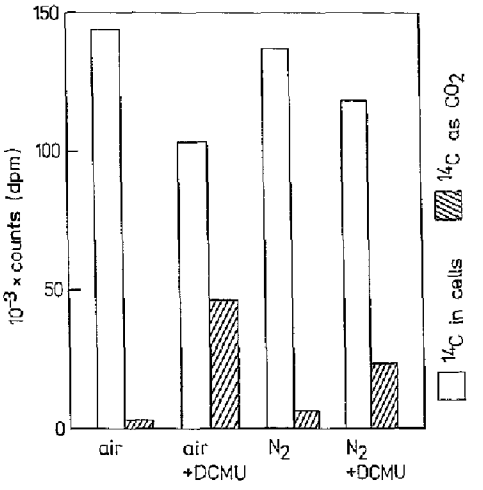

Fig. 2. Fructose uptake in the light by whole filaments of *Anabaena variabilis*. Freshly-harvested filaments were concentrated by centrifugation to 10 µg Chl/ml in fresh growth medium without fructose. At time zero 200 µM ^{14}C-fructose was added and the cyanobacteria were incubated at 30°C in the light. Reaction was terminated after 20 min by addition of TCA and [^{14}C] in cells and as CO_2 was measured as described in the Methods section

tion, and the experimental points show an excellent fit to the theoretical curve with values of K_m of 60 µM fructose and of V_{max} of 30 mmol fructose (h · mg chl)$^{-1}$. Our estimated values are very similar to the values of 50 µM fructose for the K_m and 21 mmol fructose (h · mg chl)$^{-1}$ for the maximum uptake rate reported by Haury and Spiller (1981) for the same strain of cyanobacteria adapted to fructose.

In a previously published study of dark diazotrophic growth of *A. variabilis* with fructose as carbon source (Jensen 1983) we found a growth rate of 0.0027 h^{-1} (generation time = 37 h) at an initial fructose concentration at 10 mM and a growth yield in terms of chlorophyll of 0.68 mg chl (mol fructose)$^{-1}$. Using these results a fructose uptake rate of 40 mmol fructose (mg chl · h)$^{-1}$ can be calculated. This fructose uptake rate is also close to the V_{max} for fructose of 30 mmol (h · mg chl)$^{-1}$ reported here.

Reported K_m values for cyanobacterial sugar transport are: *Nostoc* sp. ATCC 29150 (fructose) K_m = 1.2 mM (Schmetterer and Flores 1988); *Plectonema boryanum* UTEX 594 (glucose, using the glucose analogue methylglucoside) K_m = 125 µM (Raboy and Padan 1978); *Aphanacapsa* 6714 (glucose) K_m = 430 µM (Beauclerk and Smith 1978). These affinities are relatively low compared to those observed with heterotrophic bacteria and as pointed out by Smith (1983) transport mechanisms of cyanobacteria have in general lower affinity for their substrates than that of other microorganisms.

Figure 2 shows the results of an experiment in which the effects of 10 µM [3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)] on fructose uptake in the light by whole filaments of *A. variabilis* were measured under anaerobic as well as under aerobic conditions. DCMU was added to inhibit photosynthetic oxygen evolution and carbon dioxide fixation. The total fructose uptake (^{14}C in cells and ^{14}C in CO_2) in the light was independent of oxygen and DCMU. However there were large differences in the distribution of ^{14}C between filaments and CO_2. Almost no ^{14}C was found in the CO_2 fraction when the filaments were incubated in the light without DCMU. Addition of DCMU to the assay medium increases the amount of ^{14}C in the CO_2 fraction. This indicates that the CO_2 released from fructose in the absence of DCMU was refixed by photosynthesis.

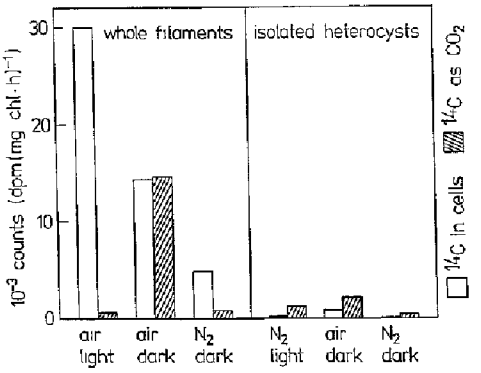

Fig. 3. Fructose uptake by whole filaments and isolated heterocytes of *Anabaena variabilis*. Fructose uptake by whole filaments was carried out as described in the legend to Fig. 2. Heterocysts were isolated in the presence of 200 mM sucrose in the isolation medium as described in the Methods section. After isolation the heterocysts were resuspended to 10 µg chl/ml in 40 mM Hepes-KOH, 1 mM $MgCl_2$, pH 7.6, supplemented with 200 mM sucrose. At time zero, 200 µM ^{14}C-fructose were added and the heterocysts were incubated at 30°C. Reaction was terminated after 20 min by addition of TCA and ^{14}C in cells and as CO_2 was measured as described in the Methods section

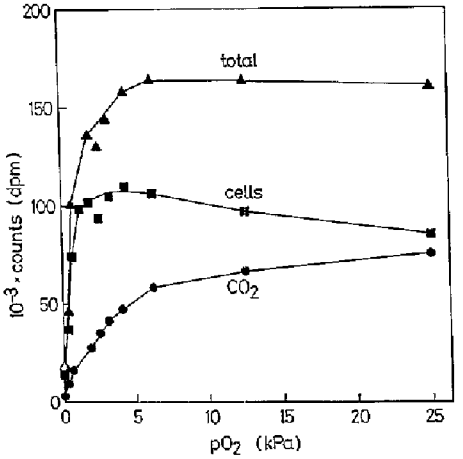

Fig. 4. Effect of oxygen on fructose uptake in the dark by whole filaments of *Anabaena variabilis*. Freshly-harvested filaments were concentrated by centrifugation to 10 µg chl/ml in fresh growth medium without fructose. At time zero, 200 µM fructose was added and the cyanobacteria were incubated at 30°C in the light. The amount of dinitrogen in the gas phase was adjusted to allow for the indicated oxygen partial pressure. Reaction was terminated afte 20 min by addition of TCA and ^{14}C in cells and as CO_2 was measured as described in the Methods section

Figure 2 also shows that much less ^{14}C was found as CO_2 if oxygen was absent from the assay medium and the experiment was carried out in the presence of DCMU. In combination with results from the experiments shown in Table 1, this suggests that some of the ^{14}C found in the CO_2 fraction was produced by oxygen-dependent respiration of fructose.

Incubation of whole filaments without oxygen in the dark significantly decreases the amount of ^{14}C found in both the cell and the CO_2 fraction (Fig. 3).

From the above results, it can be concluded that fructose uptake by whole filaments of *A. variabilis* can take place at the same rate in the dark as in the light, provided that sufficient oxygen is present. The results also show that some of the fructose carbon taken up is released as CO_2 due to respiration and suggest that most of the CO_2 released during respiration in the light is refixed by photosynthesis. Net conversion of fructose to CO_2 also occurred in the light, when refixation was inhibited, although at a slower rate than in the dark (only 33% of the fructose taken up in the light in the presence of DCMU was released as compared to 50% in the dark). This is in agreement with the well-known phenomenon of light-inhibition of respiration (Kok effect).

Investigation of fructose utilization by isolated heterocysts

We have previously described a method by which we have been able to isolate heterocysts from fructose-grown filaments of *A. variabilis* with high stability and with high acetylene-reduction capacity at the expense of endogenous reductants (Jensen et al. 1986). These heterocysts have also been shown to have a high respiration rate (unpublished results) and the capacity to perform acetylene-reduction in the dark in the presence of oxygen (Jensen et al. 1986).

To test if such heterocysts were able to take up ^{14}C-labelled fructose, the experiment shown in Fig. 3 was performed. Heterocysts were isolated with 200 mM sucrose in the isolation medium as described in the Materials and methods section. After isolation the heterocysts were suspended in 40 mM Hepes-KOH, 1 mM $MgCl_2$, pH 7.6, supplemented with 200 mM sucrose.

As shown in Fig. 3 almost no ^{14}C from fructose could be detected either in the isolated heterocysts or in the CO_2 fraction when the heterocysts were incubated in the light or in darkness with or without oxygen.

Effect of oxygen on fructose utilization in the dark by whole filaments of Anabaena variabilis

Although the isolated heterocysts used to study fructose uptake were shown to have high metabolic activity, the possibility remains that their failure to take up fructose was due to damage during isolation. We have previously shown that N_2-grown filaments of *A. variabilis* have O_2 uptake activities with both a high and a low affinity for O_2, and suggested that the high affinity activity resides in the vegetative cells and the activity with the low affinity in the heterocysts (Jensen and Cox 1983). The presence of a high and a low affinity activity for respiration in vegetative cells and heterocysts, respectively, should make it possible to distinguish between fructose utilization dependent on the metabolism of the two cell types, since fructose utilization in the dark by the vegetative cells should be saturated at low oxygen tensions while a possible fructose utilization dependent on heterocyst metabolism should require higher oxygen tensions. This approach was tested in the experiment shown in Fig. 4.

Table 2. Effect of inhibitors on ^{14}C incorporation in *Anabaena variabilis*

	Assay conditions	^{14}C in cells (% of control)	^{14}C in CO_2 (% of ^{14}C in cells)	C_2H_2 reduction (% of control)
None	light	100	2	100
10 µM CCCP	light	28	N.D.	0
50 µM OCCO	light	12	N.D.	5
1 mM EGTA	light	100	N.D.	N.D.
None (control)	dark	100	110	100
10 mM KF	dark	97	N.D.	N.D.
1 mM KCN	dark	7	N.D.	N.D.
50 µM CCCP	dark	14	N.D.	0
Anoxic	dark	15	2	N.D.

N.D.: not done

As shown, fructose uptake (^{14}C in cells + ^{14}C as CO_2) was saturated at a pO_2 of about 5 kPa and remained stable when the pO_2 was increased further. The ^{14}C in the cell fraction, on the other hand, was saturated at a lower pO_2 (about 2 kPa) and decreased when the pO_2 was increased above 5 kPa, while the ^{14}C in the CO_2 fraction was not saturated even at 25 kPa O_2.

As a result of respiratory oxygen uptake by the cyanobacteria, the actual dissolved oxygen concentration in the experiment shown in Fig. 4 will be lower than the value corresponding to the pO_2 value of the gas phase, especially at low pO_2 levels. Taking this into acount, it can be concluded that the fructose uptake by whole filaments of *A. variabilis* is saturated at a very low oxygen tension and that no stimulation could be detected by increasing the pO_2 further. This suggests that all the fructose is transported into the vegetative cells and that heterocysts do not take up fructose directly from the surrounding medium. However, the effects of higher O_2 concentration on the distribution of label between CO_2 and cellular material suggest that some of the fructose taken up by the vegetative cells is transported (either as fructose or as another compound or compounds) from the vegetative cells into the heterocysts where at least some of it is transformed into CO_2 by respiration.

That fructose first enters the vegetative cells before it is transported to the heterocysts has also been shown by Haury and Spiller (1981). They showed that when fructose-adapted and -unadapted filaments of *A. variabilis* were treated with [^{14}C]-fructose and then studied by autoradiography, then the silver grains appeared first over and near the vegetative cells and later became concentrated within the heterocysts.

Effects of inhibitors on fructose uptake

Table 2 show that fructose uptake in the dark by *A. variabilis* is not affected by fluoride, which inhibits PEP-dependent translocations, but inhibited by DCCD (which inhibits proton-translocating ATPases), the uncoupler CCCP and the respiratory inhibitor cyanide. Uptake was also inhibited under anoxic conditions. These results suggest that uptake in the dark requires ATP generated by respiratory electron transport.

Table 3. Effect of ionophores on ^{14}C uptake and acetylene reduction by *Anabaena variabilis*

Ionophore	^{14}C in cell (% of control)	^{14}C in CO_2 (% of ^{14}C in cells)	C_2H_2 reduction (% of control)
None (control)	100	2	100
5 µl/ml A23187	100	N.D.	100
5 µM monensin	102	9	42
50 µM gramicidin	106	4	66
50 µM valinomycin	114	2	100
50 µM valinomycin +20 mM KCl	62	2	45
50 µM nigericin	57	2	3
50 µM valinomycin +50 µM nigericin +20 mM KCl	25	7	0

N.D.: not done

The very low level of fructose uptake under anoxic dark conditions is in agreement with the suggestion that fermentative metabolism is very inefficient in cyanobacteria (Doolittle 1979; Smith 1982).

Photosynthesis did not enhance fructose uptake over that found under dark aerobic conditions. In cyanobacteria, DCMU inhibits photosystem II but photophosphorylation linked to cyclic electron transport around PSI remains possible. DCMU was found not to have any effect on fructose uptake in the light (Fig. 2), nor did anaerobic conditions have any effect on fructose uptake in the light both in the presence or absence of DCMU (Fig. 2). However CCCP strongly inhibited fructose uptake in the light (Table 2). Thus it can be concluded that ATP produced as a result of cyclic photophosphorylation in the thylakoid membranes is sufficient to drive fructose uptake.

Effect of ionophores on fructose utilization

To provide further information about the effects of the components of the proton motive force on fructose utilization, the effects of ionophores were investigated. Valinomycin in the presence of K^+ collapses the electrical

component of the pmf, whilst the chemical component can be collapsed by nigericin, which mediates the electroneutral exchange of K^+ for H^+. Both these ionophores inhibited the utilization of fructose to about 60% of the control rate (Table 3) and the combination of the two caused a much greater inhibition. However the effects on fructose uptake were much less than those on acetylene reduction, where complete inhibition was observed in the presence of nigericin alone, in agrement with the report of Hawkesford et al. (1981).

Bacteria usually do not require calcium for heterotrophic growth, however the strain of *A. variabilis* used in this study has been reported to depend on calcium ions for dark heterotrophic growth with fructose as carbon source (Lockau 1984). Our finding (Table 3) that neither the Ca^{2+} chelator EGTA nor the divalent cation ionophore A 23187 had any effect of fructose uptake by whole filaments of *A. variabilis*, strongly suggests that Ca^{2+} is not directly involved in fructose utilization by the cyanobacterium.

Monensin, an ionophore for Na^+/H^+ exchange, had no effect on fructose uptake. There is thus no evidence for the involvement of Na^+ in fructose uptake in *A. variabilis*.

Effect of fructose analogs on fructose uptake

A range of potential analogs for fructose were tested for their capacity to inhibit fructose uptake by whole filaments of *A. variabilis*. L-arabinose, D-arabinose, fructose-6-phosphate, fructose-1-phosphate, deoxyribose and glucose at concentrations of 1 and 10 mM and sucrose at concentrations up to 200 mM did not affect fructose uptake. The same was the case for erythrose at a concentration of 1 mM. However, 10 mM erythrose caused a decrease in the fructose uptake to about half of that found in the control experiment (results not shown).

We have previously shown that erythrose was able to double the oxygen uptake rate by whole filaments of this strain although it was unable to stimulate the rate of diazotrophic growth (Jensen 1983).

Erythrose has also been shown to stimulate acetylene-reduction by isolated heterocysts from photoautotrophically-grown *Anabaena* 7120 (Privalle and Burris 1984) and *A. variabilis* ATCC 29413 (Jensen et al. 1986). On the other hand erythrose was shown to inhibit acetylene-reduction by heterocysts isolated from filaments of *A. variabilis* ATCC 29413 grown photoheterotrophically with fructose as carbon source (Jensen et al. 1986) and also to inhibit H_2 stimulated acetylene reduction by heterocysts isolated from photoautotrophically grown *Anabaena* 7120 (Privalle and Burris 1984).

No explanation is available for these surprising results. However, it is evident that high concentrations of erythrose affect fructose uptake and heterocysts metabolism in *A. variabilis*.

The finding that erythrose at a concentration of 10 mM (200 times higher that the K_m for fructose) was the only compounds able to inhibit fructose uptake, in combination with observations that none of the carbohydrates glycerol, ribose, glucose, mannitol, gluconate, sucrose, maltose or erythrose (results not shown) were able to support photoheterotrophic growth of *A. variabilis*, strongly suggests that the transport system of *A. variabilis*, for fructose to be very specific.

Concluding remarks

The results presented here suggest that fructose utilization by this strain of *Anabaena variabilis* depends on uptake by the vegetative cells in a process which depends on the existence of a transmembrane pmf. Even isolated heterocysts with high metabolic activity cannot take up exogenous fructose; however there is evidence that labelled carbon can be transported to the heterocysts and released as CO_2 as the result of heterocyst respiration. The fructose transport system has a relatively low affinity and seems to be very specific for its substrate; its function in the natural environment is a mystery.

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