Exchange of Metabolites in *Cyanophora paradoxa* and its Cyanelles*

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Received: January 15, 1990; Accepted: April 14, 1990

Abstract

The flagellate *Cyanophora paradoxa* contains blue-greenish, organelle-like inclusions termed cyanelles which perform photosynthetic CO_2-fixation in place of chloroplasts. By the use of the HPLC-technique, *Cyanophora* was shown to form glucose, sucrose, maltose, mannitol, ribose, glycerol and trehalose. Extracts from the whole organism and from the eucaryotic host, but not from the cyanelles, convert ^{14}C-labelled UDP-glucose to polyglucan. Synthesis of sucrose from UDP-glucose and fructose-6-P or fructose could not be demonstrated in any extract from *Cyanophora*. The transfer of metabolites into cyanelles was monitored by the silicone oil filtering technique. The solute spaces for ^{14}C-labelled sorbitol and ^3H_2O were the same indicating that sorbitol freely penetrated the plasma membrane of cyanelles in contrast to the situation found in chloroplasts. The measurements of the solute spaces for the different compounds showed that maltose and sucrose were not accumulated by isolated cyanelles. Other compounds like fructose, fucose, glutamine or glycine had intermediate sizes of their solute spaces. Cyanelles apparently possess a rapidly transporting glucose carrier and not a malate/oxaloacetate shuttle and also not an ATP/ADP translocator. The carrier composition at the plasma membrane of cyanelles and at the inner envelope membrane of chloroplasts seems to be totally different.

Key words

Cyanelles, *Cyanophora paradoxa*, endosymbiosis, silicone oil layer centrifugation, metabolite exchange, polyglucan synthesis, sucrose synthesis.

Abbreviations and Symbols

chl. a:	chlorophyll a
HPLC:	high performance liquid chromatography

Introduction

The photoautotrophically growing flagellate *Cyanophora paradoxa* contains 2–4 blue-greenish, organelle-like structures (the cyanelles) which have functions analogous to chloroplasts of higher plants. *Cyanophora paradoxa* and a few other organisms containing cyanelles are classified as glaucocystophyta (Kies and Kremer, 1986) which likely is an old taxon of unresolved affinities. The glaucocystophyta have attracted special attention in the past because the cyanelles have been regarded as evolutionary models for the acquisition of plastids in eucaryotes. The cyanelles share features with both unicellular cyanobacteria and chloroplasts (see Wasmann et al., 1987; Reisser, 1984; Sitte and Hansmann, 1986; Melkonian, 1989).

As cyanobacteria, cyanelles have a cell wall which, however, consists only of the inner peptidoglycan layer of Gram-negative bacteria (Schenk, 1970; Herdman and Stanier, 1977; Giddings et al., 1983). This rudimentary cell wall contains penicillin-binding proteins (Berenguer et al., 1987) and is indispensable for the division of cyanelles (Berenguer et al., 1987; Kies, 1988). Both genes for the subunits of ribulose1-5 bisphosphate carboxylase are encoded and co-transcribed on the cyanelle chromosome (Heinhorst and Shively, 1983; Starnes et al., 1985; Mangeney and Gibbs, 1987), whereas in plants the small subunit is nuclear-encoded.

Cyanelles also have features in common with chloroplasts. The genome size is only 5–10% of that of free-living cyanobacteria and comparable with that of chloroplasts (Herdman et al., 1979; Mucke et al., 1980). The location and transcription of the *atpB*-gene (coding for the β-subunit of the ATP synthase CF_1-complex) is the same in chloroplasts and different in cyanobacteria (Wasmann et al., 1987). As in photosynthetic cells of higher plants, nitrate reduction in *Cyanophora* is light dependent (Bothe and Floener, 1978), and the location of the enzymes involved is the same. Nitrate reductase is in the cytoplasm of

Bot. Acta 103 (1990) 392–398

* Dedicated to Professor André Pirson on the occasion of his 80th birthday.

higher plants and in the eucaryotic host of *Cyanophora* (Floener and Bothe, 1982; Boettcher et al., 1982), and nitrite reductase is chloroplastic or cyanelle bound, respectively (Floener et al., 1982). Cyanelles do not respire (Floener and Bothe, 1982). Such characteristics exclude growth of cyanelles independently of the host.

Starch granules are only found in the host (Schnepf et al., 1966; Kies, 1979) indicating that the eucaryote must have effective sinks for photosynthate from the cyanelles. Very little is known about the transfer of metabolites from the cyanelles to the eucaryotic host. Long-term $^{14}CO_2$-labelling experiments with intact cells gave indirect indications that glucose (Kremer and Kies, 1979) or glucose plus a disaccharide (presumably sucrose) (Trench et al., 1978) could be the transport forms for photosynthate from the cyanelles.

In the present investigation, the primary products of CO_2-fixation were reinvestigated using HPLC for the separation of the compounds formed. The utilization of ^{14}C-UDP-glucose was also followed in extracts from *Cyanophora*. In addition, the uptake of labelled compounds into cyanelles was determined using the silicone layer filtering centrifugation technique.

Materials and Methods

Growth of the organism

The Pringsheim strain of *Cyanophora paradoxa*, LB 555 UTEX, was originally obtained from Prof. L. Provasoli (Yale University, New Haven, Conn. USA) and was grown essentially as previously (Bothe and Floener, 1978). However, the concentration of KNO_3 was increased to 1×10^{-2} M and that of $MgSO_4$ to 4×10^{-3} M (see Marten et al., 1981). *Cyanophora* was grown at 25 °C and 10,000 lux under continuous gassing with a mixture of filtered air/5 % CO_2 (v/v) in 250 ml tubes placed into a culture apparatus described by Lorenzen (1959) and manufactured by Edwards-Kniese (Marburg-Marbach, FRG). The culture was diluted 1 : 3 with fresh sterile medium three times a week, and the generation time was approximately 24 h under these conditions (Kaufmann, 1987).

Table 1 Sugar content of *Cyanophora paradoxa* determined by HPLC.

Retention time (s)	sugar identified	concentration of the sugar µg/mg chl.	area/ mg chl.
320			9 000
380			32 600
430			146 400
590	Maltotetraose	2.5	17 100
630	Sucrose	6.7	31 600
660	Maltose	2.3	10 800
705			72 400
760	Glucose	4.8	23 500
1140	Glycerol	7.8	31 800
1220	(myo-)Inositol	1.8	8 100
1550	Mannitol	1.8	8 200
1850	Ribose	0.2	900

Sugars were isolated from the cells by extraction with boiling methanol followed by ethyl acetate extraction and treatment with Dowex-50 and then with Dowex-1 to remove charged molecules. Sugars from the eluate of the Dowex-1 column were then separated from each other by HPLC using an AMINEX HPX-87P column (Biorad, München, FRG) and quantified refractometrically. To estimate the concentrations of the unidentified substances, arbitrary units were defined for the signals from the integrator coupled to the refractometer.

Determinations of the sugars

The sugars indicated in Table 1 were isolated essentially as described by Schmitz and Holthaus (1986). 600–1000 ml culture with a protein content of approximately 1 mg/ml was centrifuged, washed twice with distilled H_2O, and the cells were boiled in 80 % ethanol for 10 min. Denatured protein was removed by centrifugation. The supernatant was evaporated to dryness. The dried material was resuspended in 5 ml H_2O and pigments were removed by extraction, first with ethyl acetate and then with chloroform (both 3–4 times). Acidic and basic components were removed by ion exchange chromatography using Dowex 50 WX-8 and Dowex 1 X-8 resins (Splittstoesser, 1969). The neutral fractions containing soluble carbohydrates were separated by HPLC using an Aminex HPX-87 P, 300 × 7.8 mm column (Bio-Rad, München, F.R.G.) operating at 85 °C and with distilled H_2O as the mobile phase (flow rate 0.5 ml/min). The separated sugars were quantified by a refractive index detector (ERC-7510, Erma, Tokyo, Japan) coupled to a recording integrator system.

Polyglucan synthesis in extracts from Cyanophora paradoxa

As explained under Results, polyglucan synthesis was followed by the incorporation of UDP-[^{14}C]-glucose using the assay conditions for sucrose-6-P synthetase (EC 2.4.1.13) or sucrose synthetase (E.C. 2.4.1.14) described by Salerno et al. (1979). The assay for sucrose-6-P synthetase (see Table 2) was performed in Eppendorf tubes containing, in a final vol. of 0.1 ml: 1 µmol UDP-glucose, labelled with 0.1 µCi UDP-[^{14}C]-glucose, 2 µmol NaF, 5 µmol Hepes-NaOH buffer pH 6.5, – where indicated – 1 µmol fructose-6-P, and crude extract from *Cyanophora* with 0.025–0.05 mg protein. The incubation was performed for 20 min at 30 °C and terminated by adding 10 µl glycine/NaOH-buffer pH 10.0 and by boiling for 10 min. After cooling, the sample was incubated with alkaline phosphatase (1 unit/sample, 20 min, 37 °C) to remove phosphorus from sucrose-6-P or any other phosphorylated carbon compound formed. This incubation was stopped by adding 0.2 ml of ice-cold water and centrifugation.

Table 2 Incorporation of UDP-[^{14}C]-glucose by extracts from *Cyanophora paradoxa*.

Experimental condition	specific activity (µmol/h × mg protein)
1. *under the assay conditions for the synthesis of sucrose-6-P*	
a) complete	5.1
b) + fructose-6-P	6.7
c) extract boiled	0.2
2. *under the assay conditions for the synthesis of sucrose*	
a) complete	4.3
b) fructose	4.1
c) extract boiled	0.3

3. *UDP-glucose incorporation into homogenates from intact cells, from the cyanelles and from the eucaryote*	total protein (mg)	total activity (µmol/h)	specific activity (µmol/h × mg protein)
a) from intact *Cyanophora*	48.6	497.4	10.3
b) from the cyanelles	29.0	30.1	1.1
c) from the eucaryote	27.7	471.4	17.5

The assay conditions for the sucrose-6-P synthetase, the sucrose synthetase and the incorporation of UDP-[^{14}C]-glucose in homogenates from intact *Cyanophora*, from the cyanelles and from the eucaryote are described in Materials and Methods.

Table 3 Solute-spaces of isolated cyanelles.

Substance	isolation medium with 330 mM sorbitol		isolation medium with 330 mM sucrose	
	solute space (µl/mg chl.)	$S_H - S_C$ (µl/mg chl.)	solute space (µl/mg chl.)	$S_H - S$ (µl/mg chl.)
^3H_2O (= S_H)	88.2 ± 5.2	---	127.2 ± 9.1	---
sorbitol	83.8 ± 4.3	4.4 ± 3.1	122.5 ± 3.4	4.7 ± 3.2
sucrose	61.9 ± 2.4	26.5 ± 2.4	105.3 ± 4.1	22.0 ± 4.0
maltose	59.5 ± 5.9	28.8 ± 4.8	101.8 ± 6.7	25.9 ± 6.9
D-glucose	80.7 ± 3.2	7.5 ± 3.0	120.4 ± 4.5	6.8 ± 4.6
L-glucose	78.6 ± 4.5	10.4 ± 4.5	112.2 ± 8.7	15.0 ± 8.3
fructose	78.4 ± 4.6	14.8 ± 4.1	113.5 ± 4.5	13.8 ± 4.5
glycerol	80.2 ± 3.7	8.0 ± 3.4	115.6 ± 5.9	11.5 ± 5.8
fucose	78.4 ± 6.6	9.9 ± 6.5	106.0 ± 8.3	21.3 ± 7.9
myo-inositol	79.9 ± 4.2	8.3 ± 4.0	120.7 ± 3.0	6.6 ± 3.2
malate	57.8 ± 3.9	29.9 ± 3.9	89.3 ± 5.6	37.9 ± 5.2
malate + unlabelled oxaloacetate	52.4 ± 6.0	28.4 ± 4.2	89.0 ± 4.7	38.2 ± 4.5
glycine	73.7 ± 3.5	8.7 ± 3.5	117.4 ± 2.6	9.8 ± 2.4
glutamate	64.6 ± 3.4	23.7 ± 3.7	---	---
glutamine	73.9 ± 4.0	14.3 ± 5.1	---	---
ATP	58.2 ± 5.3	26.7 ± 6.4	91.7 ± 8.7	35.5 ± 8.2
ADP	55.7 ± 5.5	32.0 ± 5.8	94.0 ± 11.2	33.2 ± 10.2

S_H = solute space for ^3H_2O, S_C = solute space for the substance to be determined. Standard deviations were calculated from at least 16 different determinations (from at least 4 different experiments). The data were taken from an incubation time of 1–2 min. For other experimental details see Materials and Methods.

For measuring sucrose synthetase activity, the tubes contained in a final vol. of 0.1 ml: 1 µmol UDP-glucose, labelled with 0.1 µCi UDP-[^{14}C]-glucose, – where indicated – 1 µmol fructose, 5 µmol Tris-HCl-buffer, pH 8.0 and crude extract with 0.025–0.05 mg protein. The incubation was for 20 min at 30 °C and was terminated by boiling and centrifugation. After cooling, 0.2 ml H_2O was added.

In both assays, non-incorporated UDP-glucose was bound onto a Dowex 1 X 8, 200–400 mesh column, and the radioactivity eluting from the column was quantified by liquid scintillation spectrometry. To identify the reaction products of either assays, the radioactive compounds eluting from the Dowex column were separated from each other by HPLC using an Aminex HPX-87 P column or by thin layer chromatography (see Lewis and Smith, 1967). The radioactive compounds eluting from the Dowex column were also incubated with α-amylase and α-glucosidase (both from Boehringer) to degrade any polyglucan formed. The reaction products were separated from each other by thin-layer chromatography and identified by scanning of the radioactivity.

Both sucrose-6-P and sucrose synthetase activities (see lines 1 and 2 of Table 2) were measured in crude extracts that were obtained by centrifuging 200 ml culture (with about 1–2 mg chlorophyll in total) and by suspending the pellet in a buffer containing 5 mM Tris/HCl pH 8.0, 5 mM EDTA and 10 mM NaCN (Salerno et al., 1979). The cells were passed twice through a French Press at 140 000 kPa followed by centrifugation to remove debris.

For determining the distribution of the UDP-[^{14}C]-glucose incorporation activity among cyanelles and eucaryote (see line 3 of Table 2), *Cyanophora* (600 ml culture with 3–6 mg chlorophyll in total) was centrifuged (5 min, 20 000 g at 4 °C) and suspended in 10 ml buffer containing 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$ and 10 mM NaCN. The cells were gently passed twice through the French Press at 3 500 kPa. One third of this preparation was then passed twice through the Press at 140 000 kPa and centrifuged (20 min, 45 000 g) to obtain a crude extract of both the eucaryote and the cyanelles. The remaining 2/3 of the preparation was centrifuged (20 min, 750 g). The supernatant was free of cyanelles and was used as extract from the eucaryote. The pellet containing mainly unbroken cyanelles was suspended in 5 ml buffer, passed twice through the French Press at 140 000 kPa, centrifuged (20 min, 45 000 g) and used as extract from the cyanelles.

Purification of the cyanelles by Percoll centrifugation and silicone filtering centrifugation

For studying the exchange of metabolites between cyanelles and eucaryote (see Table 3), the silicone oil filtering centrifugation technique developed by Klingenberg and Pfaff (1967) for mitochondria, and by Heldt and Sauer (1971) for chloroplasts was adapted to the present purpose. Such a study required the purification of isolated cyanelles by Percoll centrifugation. 1000 ml culture with 5–10 mg chlorophyll in total was centrifuged (5 min, 650 g, 4 °C), resuspended in fresh medium and centrifuged once more. The pellet was then resuspended in 25 ml of a buffer containing 330 mM sorbitol (or sucrose), 50 mM Tricine-KOH pH 8.0, 2 mM EDTA and 1 mM $MgCl_2$ and was passed three times through a prechilled French Press at 3500 kPa. This procedure broke almost all the *Cyanophora* cells but left most of the cyanelles intact. The cyanelles were spun down (5 min, 650 g), gently suspended in the sorbitol-buffer and centrifuged (10 min, 1600 g) through a layer of 40 % Percoll suspended in the same concentration of the sorbitol-buffer using conical Falcon tubes and a Heraeus Christ minifuge 2. Cyanelles from the bottom of the tubes were suspended and washed with the sorbitol-buffer.

The cyanelles were immediately incubated for 5 min in a water bath at 25 °C, and the reaction was started by adding the different carbon compounds (see Table 3). The final concentration of each of the carbon compounds was 20 mM, and the radioactive labelling was 0.12 µCi for the carbon compounds and 0.2 µCi for ^3H_2O/assay. After the incubation time indicated in the figures (see Fig. 1 and 2), the samples were centrifuged through the silicone oil layer (see Heldt, 1980) using a Beckman Microfuge E and 400 µl polyethylene tubes containing 20 µl 1.2 M $HClO_4$ (at the bottom), 70 µl silicone oil (AR 200, Wacker Chemie, München) and

200 µl suspension of cyanelles with 15–25 µg chlorophyll in total (on top). After the centrifugation the radioactivity, both in the supernatant above the silicone layer and in the $HClO_4$-phase containing the pellet, was determined by scintillation counting.

Other methods

Chlorophyll a was determined in 80% acetone with sonicated cyanelles using an extinction coefficient of 82 mM^{-1} cm^{-1} commonly taken for cyanobacterial chlorophyll a (Biggins, 1967). Protein was measured by the Bradford method.

Results

In previous investigations, sugars from *Cyanophora paradoxa* had been separated from each other by paper or thin layer chromatography (Trench et al., 1978, Kremer et al., 1979). In the current study, separation of the sugars was performed by HPLC using an Aminex HPX-87 P column. The chromatogram showed 12 distinct peaks 8 of which were tentatively identified by their retention times and by comparison with those of standards (Table 1). Under the conditions employed, the following compounds eluted from the column: maltotetraose 580 s, sucrose 630 s, maltose 660 s, glucose 760 s, glycerol 1140 s, inositol or myo-inositol 1220 s, mannitol 1550 s and ribose 1850 s. To obtain an estimate for the relative concentrations of the different compounds, their peak areas were integrated. The areas of the 4 compounds with retention times of 320 s, 380 s, 430 s and 705 s which remained unidentified amounted to approximately 65% whereas the identified compounds accounted for 35%. The elution characteristics of the Aminex column suggested that the compounds with short retention times (320 s, 380 s and 430 s) might be oligosaccharides. The substance with a retention time of 705 s was neither stachyose, raffinose, lactose, xylose, sorbose, galactose, rhamnose, fructose, fucose, mannose, arabinose, mannitol or sorbitol as deduced from the elution pattern of standards with each of these compounds. The fractions containing glycerol, glucose, maltose and sucrose were further analyzed for their content by enzymic assays. The glucose and maltose fractions amounted to 100% of each of these compounds, whereas the fraction with a retention of 1140 s consisted of only 20% glycerol and that eluting with 630 s of 30–40% sucrose. Cellobiose and trehalose had the same retention time as sucrose. Cellobiose is a constituent of the plant cell wall and is unlikely to be found in *Cyanophora*. Trehalose occurs in many microorganisms and is, therefore, also a good candidate for *Cyanophora*. The other compound(s) of the glycerol fraction remained unidentified.

In photosynthetic cells of higher plants sucrose synthesis proceeds outside the chloroplasts. To find whether the situation is analogous in *Cyanophora*, the incorporation of UDP-[^{14}C]-glucose by extracts from *Cyanophora* was determined. Table 2 shows that UDP-glucose utilization was only slightly stimulated by fructose-6-P under the assay conditions for the sucrose-6-P synthetase. No stimulation by fructose was obtained in the test for sucrose synthetase. The same results in both assays were obtained when UDP-glucose was substituted by ADP-glucose (data not shown). After termination of the UDP-glucose incorporation experiments, attempts were made to separate the reaction products by HPLC or thin layer chromatography. All the radioactivity incorporated from UDP-[^{14}C]-glucose was recovered in a single broad spot which rapidly eluted from the Aminex column and remained at the start of the thin layer plates. The compound(s) of the spot could, to a large extent, be degraded by adding α-amylase and α-glucosidase indicating that it consisted of polysaccharide(s). The incorporation of UDP-[^{14}C]-glucose is performed by the eucaryotic host (Table 2). The cyanelles had only about 5% of the activity which was probably caused by contamination of the extract of the eucaryote in the cyanelle fraction. It had previously been communicated that only the eucaryote in *Cyanophora* stores polysaccharides (see Wasmann et al., 1987).

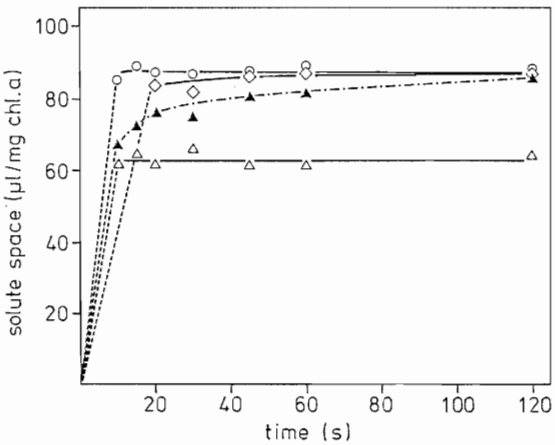

Fig. 1 The uptake of water, sucrose, sorbitol and glucose into isolated cyanelles.
The abscissa indicates, in s, the incubation time of cyanelles with radioactive labelled compounds which were:

○—○ ^3H_2O
△—△ sucrose
▲–·–▲ sorbitol
◇—◇ glucose

The latter three compounds were uniformly ^{14}C-labelled. For other experimental details see Materials and Methods.

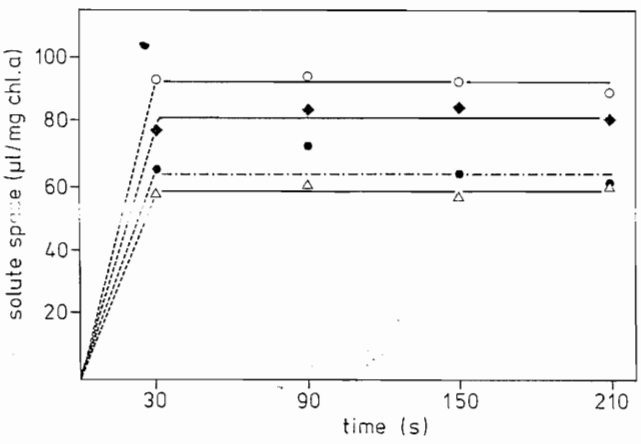

Fig. 2 The uptake of fructose and maltose into cyanelles. The ^3H_2O- and sucrose-spaces were added for comparison.

○—○ ^3H_2O
◆—◆ fructose
●–·–● maltose
△—△ sucrose, the latter three uniformly ^{14}C-labelled

The uptake of metabolites into cyanelles was investigated by the silicone oil filtering centrifugation technique. In chloroplasts of plants H_2O freely penetrates all membranes, and the ^3H_2O-solute space is, therefore, indicative of the total aqueous volume pelleted (Heldt and Sauer, 1971; Robinson, 1985). The outer envelope membrane of chloroplasts is permeable to all compounds of low molecular weight. The inner envelope membrane is the site of specific carrier transport and is virtually impermeable (Heldt, 1980) or very slowly penetrated by sorbitol (rate 10–20 µmol/h × mg chl., Robinson, 1985). The sorbitol-impermeable ^3H_2O-space can, therefore, be taken as a measure of the volume of the filtered chloroplasts. With regard to cyanelles, the host vacuole is destroyed during the isolation procedure. The cyanelles, however, retain the inner envelope membrane which is similar to the cyanobacterial plasma membrane (Giddings et al., 1983) and might be the transport barrier. Isolated cyanelles might retain part of the residual peptidoglycan and the lipopolysaccharide layers of Gram-negative bacteria seen in intact *Cyanophora* (Giddings et al., 1983, Wasmann et al., 1987).

As with chloroplasts (Heldt, 1976), ^3H_2O is rapidly taken up by isolated cyanelles, and the ^3H_2O-solute space no longer increases after 30 s of incubation (Fig. 1). Surprisingly, ^{14}C-sorbitol was taken up by the cyanelles almost as fast as ^3H_2O, and there was virtually no difference between the sizes of the H_2O and the sorbitol solute spaces (Fig. 1). The other compounds tested (Figs. 1–2, Table 3) were also rapidly taken up within 30–60 s at the experimental temperature of 25 °C. However, the solute spaces for maltose and sucrose (Fig. 2) amounted to about 60 µl/mg chl. and were therefore considerably lower than those for water and sorbitol. The space for glucose reached the size of the ^3H_2O- and sorbitol spaces (Fig. 1) and that for fructose was intermediate (Fig. 2). It should be noted that the differences between the spaces for ^3H_2O and sucrose (or maltose) were consistently 27–35 µl/mg chl. in each of all the different assays; irrespective of the absolute sizes of the solute spaces which showed maximal variations of 30 % from experiment to experiment. The same consistency in the differences was also observed when the other labelled compounds listed in Table 3 were tested.

Such a difference volume of 27–35 µl/mg chlorophyll could well represent the interior of the cyanelles surrounded by the inner envelope membrane which might form a barrier against the diffusion of compounds such as maltose or sucrose. However, it cannot be ruled out that the peptidoglycan layer and the residual outer membrane of the cyanelles significantly contribute to this space. In any case the isolated cyanelles used apparently preserve selectivity for the uptake of compounds.

The data of Table 3 indicate the following:

a) The spaces for glucose and ^3H_2O were almost the same, indicating that glucose is effectively taken up by the isolated cyanelles. However, in contrast to chloroplasts (Schäfer et al., 1977), cyanelles do not discriminate between D-glucose and L-glucose.
b) Cyanelles also utilize alcohols other than sorbitol, e.g. myo-inositol or glycerol.
c) The size of the space for fructose is significantly smaller than that for glucose indicating that cyanelles selectively discriminate between monosaccharides.
d) Cyanelles also discriminate between amino acids. The solute space sizes are glycine > glutamine > glutamate, and the latter is at best poorly taken up.
e) Malate is not utilized, regardless of whether it is supplied alone or with oxaloacetate. The same is true for ATP and ADP.
f) When the cyanelles were prepared with 330 mM sucrose instead of sorbitol, the absolute sizes of the spaces were increased by some 50 % with all labelled substances offered. However, the difference between the ^3H_2O and the sucrose (or maltose) spaces was again 27–35 µl/mg chl. These differences between the spaces for water and all other compounds offered were at best slightly dependent on the isolation medium used. Fucose and perhaps also malate represent an exception to this rule.

Table 4 Solute spaces of cyanelles isolated with different media.

Substance	isolation medium			
	sorbitol	K^+-malate	KCl	betaine
^3H_2O	88.2 ± 5.2	72.7 ± 5.2	67.5 ± 9.2	89.9 ± 9.5
sucrose	61.9 ± 2.4	58.0 ± 9.0	48.1 ± 5.0	64.7 ± 5.1
$S_H - S_{sucrose}$	*26.5 ± 2.4*	*16.2 ± 8.3*	*21.3 ± 5.1*	*25.2 ± 5.0*
glucose	80.7 ± 3.2	65.4 ± 5.1	62.7 ± 3.7	86.4 ± 3.7
$S_H - S_{glucose}$	*7.5 ± 3.0*	*7.2 ± 5.0*	*6.8 ± 3.5*	*3.3 ± 3.4*
fructose	78.4 ± 4.6	65.4 ± 4.7	57.4 ± 2.1	82.7 ± 4.8
$S_H - S_{fructose}$	*14.8 ± 4.1*	*7.3 ± 4.7*	*12.1 ± 2.2*	*7.2 ± 4.2*
malate	57.8 ± 3.9	56.2 ± 6.3	50.8 ± 5.0	61.2 ± 3.8
$S_H - S_{malate}$	*29.9 ± 3.9*	*16.5 ± 6.2*	*19.4 ± 4.5*	*28.7 ± 3.9*
ATP	58.2 ± 5.3	58.0 ± 5.6	52.7 ± 4.3	64.2 ± 5.0
$S_H - S_{ATP}$	*26.7 ± 6.4*	*14.6 ± 5.5*	*16.8 ± 4.3*	*26.0 ± 5.5*

S_H = solute space for ^3H_2O. In the isolation steps, sorbitol was substituted by either 200 mM KCl, K^+-malate or betaine (isolation performed at pH = 8.0 in all cases). Otherwise the components (see Materials and Methods) were not changed during the isolation procedure.

The data of Table 3 could also mean that neither sorbitol nor sucrose were optimal for isolating cyanelles. Thus other osmotica which had been used for the isolation of chloroplasts (Heldt, 1980; Robinson, 1985) were tried (Table 4). The absolute sizes of the solute spaces for all substances, and also the differences in the spaces, were small when the cyanelles had been isolated with K^+-malate or KCl. Betaine gave similar results as sorbitol. In all cases, the pattern in the absolute spaces and also in the differences was essentially the same, irrespectively of the osmoticum used for the isolation of cyanelles. The solute space for glucose had almost the same size as that for ^3H_2O, whereas malate, ATP and sucrose apparently did not, or at best poorly, penetrate into cyanelles. The size of the fructose space was intermediate but showed largest variations depending on the osmoticum used.

The uptake of metabolites was not stimulated by light. This was tested for glucose and fructose in cyanelles isolated with either sorbitol or sucrose. The solute spaces did not change upon illuminating the samples in these cases (data not shown).

Discussion

The present study confirms, by the use of the HPLC-technique, that glucose (Trench et al., 1978), sucrose (Schenk and Hofer, 1972; Trench et al., 1978), maltose (Kremer et al., 1978) and glycerol (Schenk and Hofer, 1972) are accumulation products of free carbohydrates (neutral constituents) in *Cyanophora*. Mannitol, ribose, maltotetraose, myo-inositol and trehalose (the latter probably) were now detected. Attempts to demonstrate sucrose synthesis in extracts from *Cyanophora* have so far failed. The assay conditions and the regulatory properties of sucrose synthesis may well be different in higher plants and in *Cyanophora*. On the other hand, the data of Table 2 indicate that the cytoplasm outside the cyanelles constitutes an effective sink for the conversion of UDP-glucose to polyglucan. Separation of the enzymes of sucrose formation from those of polyglucan synthesis would clarify whether sucrose synthesis proceeds outside the cyanelles, as it does in the cytoplasm of higher plants.

The data obtained with the silicone oil centrifugation technique indicate that isolated cyanelles retain their selectivity for metabolites. The uptake carriers of cyanelles are strikingly different from those of chloroplasts or mitochondria. In these plastids, conclusions on the carrier composition are drawn from studies on the uptake of metabolites, and the carriers are assumed to be freely reversible. If this also applies to cyanelles, the data indicate that the malate/oxaloacetate carrier of chloroplasts and the ATP/ADP translocator of mitochondria (see Heldt, 1976) are absent in cyanelles. Maltose is excreted by the symbiotic *Chlorella* in *Paramecium bursaria* (Ziesenisz et al., 1981), but the data for cyanelles indicate that the disaccharides, maltose and sucrose, do not (or poorly) penetrate the plasma membrane of cyanelles. On the other hand, the glucose and water space have about the same size which might be indicative of glucose being the major transport form of photosynthate from cyanelles. Such findings support earlier more circumstantial evidence for glucose transfer (Trench et al., 1978; Kremer et al., 1979). Effective glucose transfers are known for erythrocytes (Le Fevre, 1959) and for the symbiotic *Chlorella* of *Spongilla fluviatilis* (Fischer et al., 1989). A translocation of glucose was also described in chloroplasts (Schäfer et al., 1977). However, the apparent K_M of D-glucose for this carrier was 10–30 mM and therefore too high for a significant transfer of organic carbon out of the chloroplasts by a glucose carrier during photosynthesis. Glucose was even used as osmoticum for the preparation of intact chloroplasts (Walker, 1971). Cyanelles appear mainly to utilize a glucose carrier, and preliminary evidence (unpublished) suggests that the phosphate translocator is also absent, contrary to the situation in chloroplasts. The selectivity of metabolites taken up by cyanelles is indicative of carrier-mediated transfer. The data currently available do not allow any statement on active transport processes.

Isolated cyanelles have not yet been obtained in which substantial CO_2-fixation over longer incubation periods takes place (Trench et al., 1978; Floener and Bothe, 1982). This failure might be explained by the current observation that alcohols, such as sorbitol, freely penetrate into cyanelles. In the past, sorbitol or mannitol, commonly used during the isolation of chloroplasts, have also been tried with cyanelles. Other osmotica now appear to be more promising for isolating cyanelles with sustained photosynthetic CO_2-fixation. Of those tried in the present study, KCl and K^+-malate can probably be disregarded, because the sizes of the spaces were small, probably due to electrostatic interactions at the cytoplasmic membrane of cyanelles. In contrast, the absolute sizes were considerably higher with sucrose than with sorbitol. A swelling dependent on the isolation medium was described for chloroplasts (Robinson, 1985). This might also have occurred with 330 mM sucrose in the isolation medium because the use of lower concentrations of sucrose resulted in smaller absolute sizes of the cyanelles (R. Schlichting, unpublished). Betaine unlikely penetrated into cyanelles, as it gave clear-cut differences in the solute spaces and, therefore, might be promising as an osmoticum for the isolation of intact cyanelles and for future work.

A comment on the evolutionary aspects of this work is inevitably necessary. Cyanelles have been regarded as a "bridge" or "missing link" between cyanobacteria and chloroplasts (see Wasmann et al., 1987) and have been termed "almost a cyanobacterial chloroplast" (Jaynes and Vernon, 1982). More recent studies on the molecular biology have indicated that the cyanelle DNA shares sequence homologies with both the cyanobacterial and plastidal genes (Kuntz et al., 1984; Bryant et al., 1985; Ko et al., 1985; Lambert et al., 1985). However, as pointed out (Wasmann et al., 1987), such homologies could reflect more a common function than a common ancestry. The present study shows that the carrier composition at the inner envelope membrane of chloroplasts and at the plasma membrane of cyanelles is very different. It is too premature to conclude from this that cyanelles present a dead-end branch of parallel evolution (see Sitte and Hansmann, 1986) or are more related to red algae (Jaynes and Vernon, 1982; Lemaux and Grossman, 1984), cryptomonads or any other algal group. In many algal groups polyglucan is synthesized and stored outside the chloroplasts (Bisalputra, 1974) as it is also found outside the cyanelles in *Cyanophora*. The carrier composition in the plastids of these eucaryotic algae is likely also different from those of higher plants which has, however, not been investigated. Investigations of this kind could well help in clarifying the mysterious evolutionary position of the cyanelles of the glaucocystophyta.

Acknowledgements

The authors are indebted to Helgard Kaufmann and Iris Neidt for help in performing some of the experiments and to Bernhard Arnold for useful discussions. This work was kindly supported by a grant from the Deutsche Forschungsgemeinschaft.

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