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Evidence for a peptidoglycan envelope in the cyanelles of *Glaucocystis nostochinearum* Itzigsohn

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Abstract. *Glaucocystis nostochinearum* is a eukaryotic organism with "chloroplasts" that have usually been assumed to be "cyanelles" – i.e., endosymbiotic cyanobacteria. Previous attempts by others to support this assumption by demonstrating the presence of a limiting peptidoglycan envelope have been unsuccessful.

In the present study disruption of intact *Glaucocystis* cells with a glass tissue homogenizer permitted the isolation of the uniquely-shaped cyanelles. That these cyanelles were limited by a peptidoglycan-containing envelope was concluded from the following evidence: (1) stability of isolated cyanelles in distilled water as determined by the preservation of their intactness and peculiar asymmetrical shape; (2) lysozyme sensitivity as demonstrated by lysis of isolated cyanelles when treated with low concentrations of lysozyme; (3) inhibition of the lysozyme-mediated lysis by N-acetylglucosamine-2, a known competitive inhibitor of lysozyme, (4) visualization of a thin, electron dense layer between the two limiting membranes around the cyanelle, and (5) isolation and identification of the peptidoglycan-specific amino acid, diaminopimelic acid, from the cyanelles.

Key words: Algal taxonomy – Cyanelles – Diaminopimelic acid – Endosymbiotic association – Endosymbiotic cyanobacteria – *Glaucocystis* – Peptidoglycan

Glaucocystis nostochinearum Itzigsohn, a freshwater alga of indeterminate phylogenetic identity, is characterized by a cellulosic cell wall, nucleus, mitochondria, vacuoles, dictyosomes, starch granules, and autospore formation, all suggesting an affinity to the green algae (Chlorophyceae, Order: Chlorococcales). Non-chlorophycean characters include: (1) blue-green-pigmented "chloroplast-like bodies" (Ueda 1961) which contain chlorophyll *a*, c-phycocyanin and allophycocyanin plus phycobilisomes (Chapman 1966), and (2) extra-"chloroplastidic" starch. In addition, unusual characters include internal, rudimentary flagella (Schnepf et al. 1966) and a lacunae system of membrane-bound structures bordering the plasmalemma (Schnepf et al. 1966).

Glaucocystis was originally assigned to the Schizophyceae of Rabenhorst (1866, according to Tilden 1910) – apparently solely on the basis of its blue-green color. Other early taxonomic determinations for *Glaucocystis* include the red algae, dinoflagellates, green algae, and the sub-class, Glaucocystideae (West 1904). In view of the enigmatic taxonomic status of *Glaucocystis*, Geitler (1923) proposed that Pascher's (1914) endosymbiotic interpretation of Mereschkowsky's "chromatophoren-symbiose" theory (1905) be applied to *Glaucocystis*. According to Geitler, *Glaucocystis* represented a symbiotic association in which an apoplastidic green alga serves as host or "cyanome" to blue-green "cyanelles" – in accordance with Pascher's proposed terminology. In expressing their reservations concerning the endosymbiotic explanation, Fritsch (West and Fritsch 1927) and Korschikov (1930) offered the opinion that the blue-green "symbiont" needed to be cultured separately and demonstrated to be capable of leading an independent existence before the symbiotic view could be accepted. Sukja (1948) argued that establishment of such a symbiosis by penetration is impossible since no extracellular cellulases are known to be produced by blue-green algae which could break down the cell wall of the host cell. On this basis he reasoned that the symbiotic association occurred very early in the geologic record which resulted in the mutual dependence of host and blue-green alga. Skuja created a new taxonomic group, the phylum Glaucophyta (Skuja 1954).

In an early recognition of *Cyanophora* as an apparent endosymbiotic association involving a blue-green alga (cyanobacterium) and a flagellated host, Hall and Claus (1963) described the extensive ultrastructural similarities between the photosynthetic lamellae of the cyanelles and free-living cyanobacteria. They could find no trace of a sacculus in the cyanelles. Lefort (1965) reported similar findings for *Glaucocystis*.

The cryptic nature of the phylogenetic issue in *Glaucocystis* was re-emphasized when the biochemistry of the cyanelle cell wall was considered. In intact *Glaucocystis* cells, Holm-Hansen et al. (1965) failed to detect any diaminopimelic acid, a compound which is found only in the walls of non-Archaebacterial prokaryotes (Fox et al. 1980).

Schenk (1970) found a lysozyme-sensitive remnant cell wall in cyanelles isolated from *Cyanophora*. Schenk (1971), however, was unable to achieve lysozyme-mediated lysis of the cyanelles from *Glaucocystis*. In a second paper, Schenk and Hofer (1972) reaffirmed Schenk's earlier contention that *Skujapelta nude* (= cyanelle of *Glaucocystis*, Hall and Claus 1967) has only a "lipid-containing envelope membrane similar to that in the chloroplasts".

The resignation and frustration with which algal taxonomists have viewed *Glaucocystis* during the past few years is apparent in Lewin's (1974) statement that "not even with the help of the electron microscope have we been able

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to establish unequivocally whether its pigmented bodies are blue-green algae or plastids". Clearly, Lewin was referring to the lack of evidence for a remnant cell wall in the cyanelles of *Glaucocystis*.

The present report demonstrates that the cyanelles of *Glaucocystis* do possess a peptidoglycan envelope. Evidence includes lysozyme sensitivity of the cyanelles, electron microscopic visualization of an envelope limiting the cyanelles, and a positive assay for the presence of diaminopimelic acid. The results support the conclusion that the cyanelles of *Glaucocystis* are endosymbiotic cyanobacteria which appear to have become obligately dependent on an endosymbiotic association involving an equally dependent eukaryotic host cell.

Materials and methods

Culture conditions

Axenic cultures of *Glaucocystis nostochinearum* Itzigsohn were obtained from the Cambridge Collection of Algae and Protozoa (No 229/1, Cambridge University, Cambridge, UK). Experimental cultures were grown in cotton-plugged 500-ml bubbler tubes containing 450-ml volumes of growth medium gassed with sterile air at 24° C under 1,700 lx of continuous, cool-white fluorescent illumination. The liquid medium, devised by Gustav Görtlind (University of Uppsala, Uppsala, Sweden), was of the following composition (given per liter): 0.085 g $NaNO_3$, 0.015 g KCl, 0.027 g KH_2PO_4, 0.174 g K_2HPO_4, 0.049 g $MgSO_4$, 0.047 g $Ca(NO_3)_2 \cdot 4\ H_2O$, 0.5 g N-Z-Case (Sheffield Chemical, Union, NJ, USA), 0.005 mg vitamin B_{12}, and 1.0 ml of a micronutrient solution containing the following (given as mg in 50 ml stock): 300 mg nitrilotriacetic acid dissolved in 4 ml of 1 N NaOH, 11 mg $ZnSO_4 \cdot 7\ H_2O$, 18 mg $MnCl_2 \cdot 4\ H_2O$, 48 mg $FeCl_3 \cdot 6\ H_2O$, 17.5 mg H_3BO_3, 6.3 mg $Na_2MoO_4 \cdot 2\ H_2O$, 0.37 mg $CuSO_4 \cdot 5\ H_2O$, 0.020 mg $CoCl_2 \cdot 6\ H_2O$. The initial pH of the culture medium was 7.1.

Harvesting of Glaucocystis cells

Care was taken to ensure that all experiments were conducted with axenic cultures of *Glaucocystis*. Routine transfers, inoculation of experimental cultures, samplings, and homogenization procedures were conducted in an "EdgeGARD" laminar flow transfer hood (The Baker Co., Sanford, ME, USA). After bubbler suspension cultures had achieved mid-exponential phase growth ($1-2\times10^4$ cells/ml) the air source was removed and the suspended *Glaucocystis* cells were allowed to settle for 3–4 h. Approximately 300 ml of the medium were poured off and the remaining suspended cells plus medium were transferred to sterile 40-ml polycarbonate centrifuge tubes and centrifuged at $1,000\times g$, 2 min.

Aseptic isolation of Glaucocystis cyanelles

Approximately 2 ml of packed *Glaucocystis* cells were resuspended in 40 ml of chilled growth medium and homogenized aseptically in a 40-ml capacity glass tissue homogenizer resting in an ice bath. Following homogenization, the brei was centrifuged for 1 min at $800\times g$. The cyanelle suspension (supernatant) was poured into sterile centrifuge tubes and centrifuged again for 1 min at $1,000\times g$ at 6° C. The pellets from these low-speed spins contained mostly intact cells and were rehomogenized, with the brei subjected to a second centrifugation for 1 min at $1,000\times g$ (6° C). The supernatants from the $1,000\times g$ centrifugations were poured into a sterile collecting tube (3 cm × 20 cm) which was fitted at one end with microfilament cloth (Nitex; Tobler, Ernst, and Traber, Inc., NY, USA) having a mesh size of 10 μm and then centrifuged for 20 min at $1,000\times g$ (6° C) in sterile centrifuge tubes. The pellet contained the isolated cyanelles. Pellets from several tubes were carefully resuspended in small volumes of culture medium, consolidated, and then centrifuged for 20 min at $1,000\times g$ at 6° C.

For lysozyme-sensitivity experiments or microscopy, the pellets were resuspended in culture medium and allowed to warm to 24° C before use. Pellets used for diaminopimelic acid analysis were resuspended aseptically in a small volume of growth medium and frozen immediately.

Electron microscopy

Glaucocystis cells and isolated cyanelles were fixed in 1.5% glutaraldehyde in culture medium, pH 7.1, for 1 h. This was followed by three rinses with fresh culture medium, 20 min each. The cells or cyanelles were then post-fixed in 2% OsO_4 in culture medium for 15 h at 4° C. The material was rinsed 3 times (10 min) in culture medium before being mixed into 2% agar in warmed thick-walled centrifuge tubes. The tubes were centrifuged at $1,000\times g$ for 5 min at 24° C. The agar pellet was then removed, trimmed, and dehydrated in a 2-methoxy-ethanol series. Dehydrated blocks were then transferred to ethyl alcohol, propylene oxide, and embedded in the plastic mixture of Mollenhauer (1964).

Sections were stained with 2% uranyl acetate (30–45 min) followed by Reynold's lead citrate (4 min) and examined with a Philips EM-300 electron microscope operating at an accelerating voltage of 60 kV.

Light microscopy

A Zeiss microscope fitted with Nomarski differential interference contrast optics was used for photomicrography of *Glaucocystis* intact cells or isolated cyanelles. Lysozyme-mediated lysis was observed on a Zeiss inverted microscope.

Lysozyme treatment

Hen egg white (HEW) lysozyme (M. W. 12,930: muramidase, N-acetylmuramide glycanhydrolase, EC 3.2.1.17, Sigma Chemical Co., St. Louis, MO, USA) was used in all lysozyme-mediated lysis experiments. HEW lysozyme was freshly prepared (75 μg/ml) before each experiment in double-distilled water and did not contain EDTA. The pH of the enzyme solution was 7.1. The enzyme solution was diluted to the experimental concentrations with culture medium.

For spheroplast production from the cyanelles of *Glaucocystis*, 0.6 M sorbitol (M. W. 182.17), 0.1% bovine serum albumin, 20 mM glycylglycine, 10 mM D-isoascorbic acid, 2 mM EDTA (disodium) and 5 μg/ml lysozyme were added to the culture medium. The pH was adjusted to 7.2.

The standard lysozyme-mediated digestion (24° C) treatment was modified in separate experiments to include the

use of heat-inactivated lysozyme (80° C, 15 min) and incubation at low temperature (6° C).

Phycocyanin assay

The release of phycocyanin as a water-soluble pigment was monitored as an indication of the occurrence of lysis in the cyanelles. The maximum absorption peak of phycocyanin from *Glaucocystis* cyanelles is 620 nm.

All lysozyme-mediated digestions were performed in 5-ml test tubes at 24° C. The final volume of the incubation medium was 1.5 ml and contained known volumes of the following components which were added in the following order: (1) a suspension of freshly isolated cyanelles, (2) for appropriate experiments, either a solution of competitive inhibitor of lysozyme for experimental samples, or double-distilled water for the controls, and, (3) either lysozyme for experimental samples or double-distilled water for the controls. Test tubes were quickly sealed with parafilm and mixed by inversion 3 times (1 s) after step 2 for appropriate experiments and after step 3 for all experiments. Incubation time was defined as that interval of time elapsing between the addition of lysozyme and the completion of the filtration procedure which took approximately 10 s.

The filtration apparatus consisted of a 10-ml capacity disposable syringe attached to a "Swinnex-25" membrane filter holder (Millipore Corp., Bedford, MA, USA) loaded with a 25-mm Nuclepore filter (polycarbonate, 0.22 μm). Millipore filters were found to absorb phycocyanin. The filtrates were collected in 10-ml screw-cap centrifuge tubes, capped, and placed in an ice bath. The samples were analyzed for absorbance at 620 nm with a Beckman DU monochromator (Beckman Instruments, Fullerton, CA, USA) equipped with a Gildford Model 2220 adapter. The 620 nm values were corrected for turbidity by subtracting values obtained at 720 nm.

Competitive inhibitors

N-Acetyl-glucosamine (NAG, Sigma Chemical Co., St. Louis, MO, USA) and N-acetyl-glucosamine-2 (NAG-2) were used as competitive inhibitors for the hydrolytic activity of lysozyme. The NAG-2, obtained from Pam Bolding, Department of Biochemistry, University of California, Berkeley, was isolated from a partial acid hydrolysis of chitin (Rupley 1964).

Isolation of peptidoglycan

The method of Golecki (1977) was used for the isolation of the peptidoglycan portion of the cell wall in *Anacystis nidulans*, a small unicellular cyanobacterium used as a positive control. Golecki's procedure was modified only in the initial disruption step. *Anacystis* cells (25 – 30 mg, wet wt) were harvested from agar cultures, resuspended in 20 ml of double-distilled water containing 10 mM $MgCl_2$ and 1 mg DNAase, and disrupted in a French pressure cell (Aminco, American Instr. Co., Silver Springs, MD, USA) at 138,000 kN/m^2. The material was centrifuged (Beckman L2-65B ultracentrifuge) in polyallomer centrifuge tubes at $20{,}000 \times g$ for 10 min. The previously frozen *Glaucocystis* cyanelle samples were not subjected to the disruption step but were thawed and centrifuged at $1{,}000 \times g$ for 10 min. After the supernatants were discarded, the pellets were resuspended in 5 ml of the $MgCl_2$-DNAase solution, mixed vigorously for several minutes on a micromixer and then centrifuged at $20{,}000 \times g$ for 10 min. Following treatment of the pellets according to Golecki, they were resuspended in 5 ml of double-distilled water and then centrifuged at $150{,}000 \times g$ for 30 min. The pellets were lyophilized and stored at 4° C until needed.

Amino acid analysis

Initially, the lyophilized peptidoglycan preparations from *Anacystis* and isolated *Glaucocystis* cyanelles were hydrolyzed with 6 M HCl at 110° C for 24 h before being subjected to amino acid analysis procedures. In later analyses, however, hydrolyzed *Glaucocystis* peptidoglycan material was subjected to ascending paper chromatography as an intermediate step for separating the amino acids methionine (Met) and diaminopimelic acid (DAP) which were found to elute together in the amino acid analyzer.

Known volumes of the hydrolzyed peptidoglycan preparations were equilibrated with loading buffer at pH 5.28 and loaded onto a short (0.5 cm × 6 cm) ion-exchange resin (type A chrombeads, Technicon) column and analyzed. Subsequently, samples identical to the short column loadings were resuspended in loading buffer at pH 3.25 and loaded onto long columns (0.5 cm × 24 cm). For each long-column sample, the pH was increaed to pH 4.25 after the elution of proline and allowed to run until the complete elution of the phenylalanine peak. All analyses were conducted at 60° C.

Norleucine (Nor), a normal component of the amino acid standard, was added as an internal standard (5 mM) to all of the samples analyzed. For some experimental analyses, authentic samples of either DAP (Sigma Chemical Co., St. Louis, MO, USA) or Met (Sigma Chemical Co., St. Louis, MO, USA) were added.

A Technicon TSM Sequential Multisample Amino Acid Analyzer was used to identify the amino acids of the two peptidoglycans. Although standard analysis procedures utilized wavelengths of 470 nm and 540 nm, results of analysis at the higher wavelength were generally used due to its greater sensitivity. Only the portion of each run which includes all the amino acids that elute between threonine and norleucine is presented in the figures.

Ascending paper chromatography

One-gallon glass jars with screw-lids were used as chromatography vessels into which 150 ml of the following solvent (4:1:1; N-butyl alcohol:glacial acetic acid:double-distilled water, respectively) were introduced and allowed to equilibrate for 2 h at 24° C. Cylinders of Whatman 3MM chromatography paper (16 cm × 21.5 cm) were fashioned by stapling the 21.5-cm sides together after a horizontal line (origin) was drawn 2 cm from the edge of one of the 16-cm sides.

Glaucocystis cyanelle peptidoglycan material was subjected to 6 M HCl hydrolysis for 25 h at 110° C. While taking careful measures to keep separate the authentic standards (Met and DAP) from the *Glaucocystis* peptidoglycan sample on the origin, resuspended (double-distilled water) hydrolyzed *Glaucocystis* cyanelle peptidoglycan and 25 ng-samples of Met and DAP were spotted on the line. The spots were dried immediately with a hand-held hair dryer. The paper cylinders were placed in the chromatography vessels for 4 h.

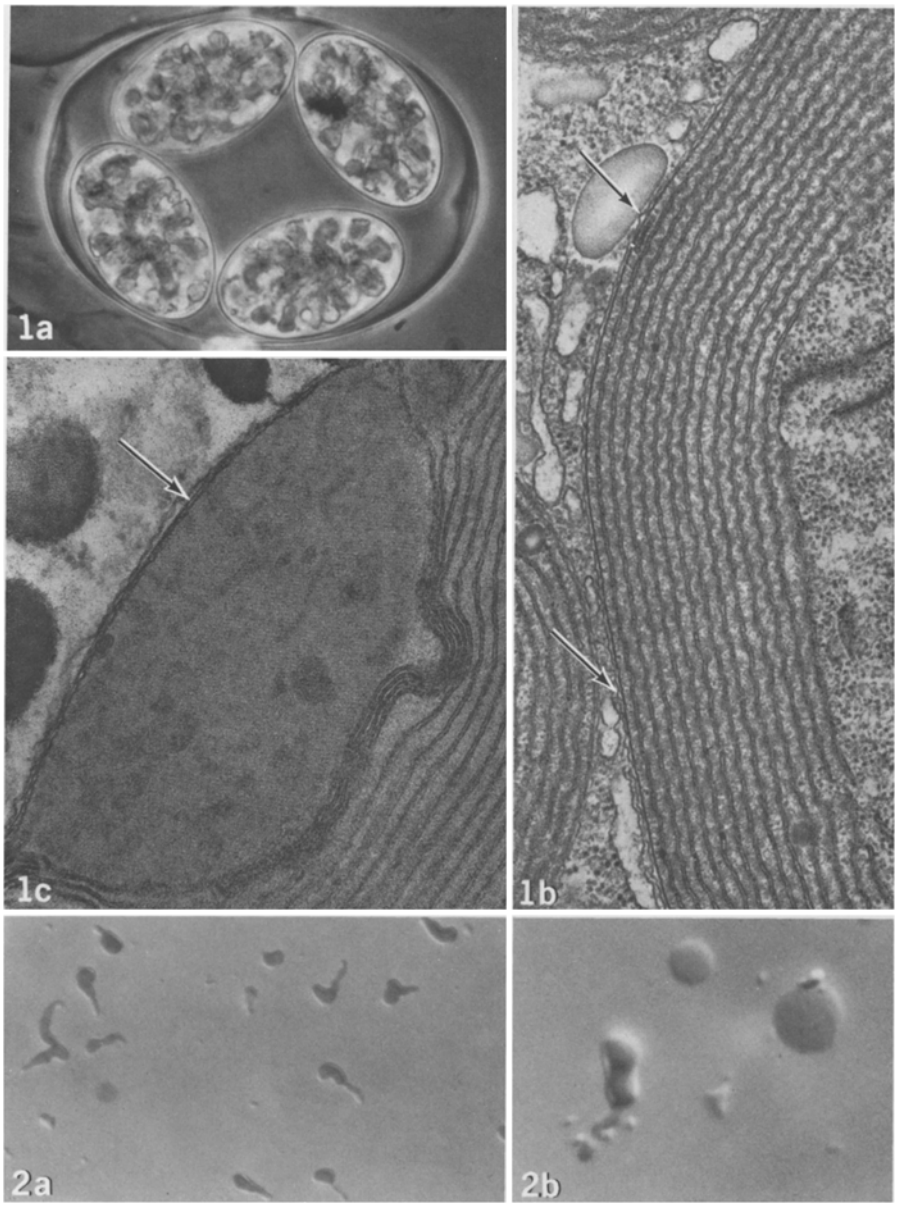


Fig. 1 a–c
Glaucocystis nostochinearum. **a** Four daughter autospores within an old autospore mother cell wall. Phase contrast, ×1,200. **b** Crenulating outer membrane surrounding the cyanelle shown at arrows. ×67,000. **c** Horizontal striations implying a crystalline substance in the lamellar-free region of an immature cyanelle. Electron-dense layer interpreted as peptidoglycan is visible at arrow, ×78,000

Fig. 2 a, b
Isolated cyanelles of *Glaucocystis*. **a** Cyanelles isolated in *Glaucocystis* culture medium (GCM), 24° C, ×1,700. **b** Spheroplast formation by cyanelles in an osmoticum containing sorbitol (0.6 M) and lysozyme (5 µg/ml), 24° C, ×2,000

After 4 h, the chromatogram was air-dried and vertical strips containing each standard were separated from the cyanelle sample. The standards were developed with ninhydrin (0.2%, aerosol) and heat-dried for 8 min at 80° C. A horizontal zone of the undeveloped portion of the chromatogram, characterized by R_f values approximating the DAP R_f value, was cut out and subsectioned into 0.5 cm^2 pieces. The pieces were eluted into 10 ml of double-distilled water. The aqueous solution was filtered through a 0.22 µm Nuclepore filter, lyophilized, and then subjected to amino acid analysis.

Results

Cell structure of the host cell in Glaucocystis nostochinearum

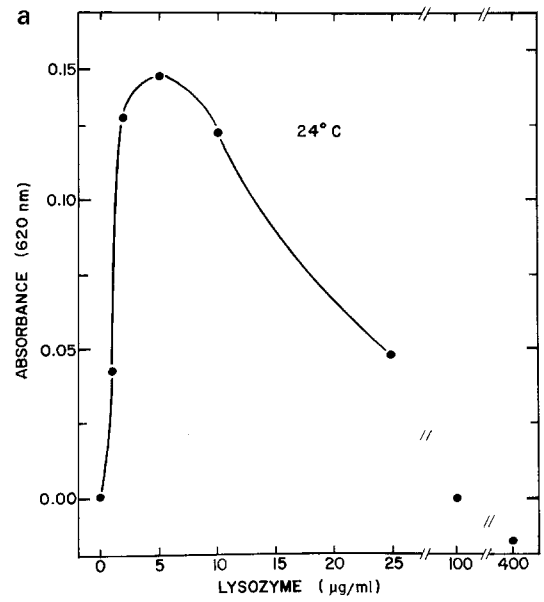
The eukaryotic host cells in *Glaucocystis* are 15–20 µm × 25–50 µm and by their oval shape bear resemblance to the green alga *Oocystis* (Schnepf 1965). The morphological resemblance is extended to autospore formation, an asexual process in which daughter autospores form within an old autospore mother cell wall after a series of mitotic divisions (Fig. 1 a).

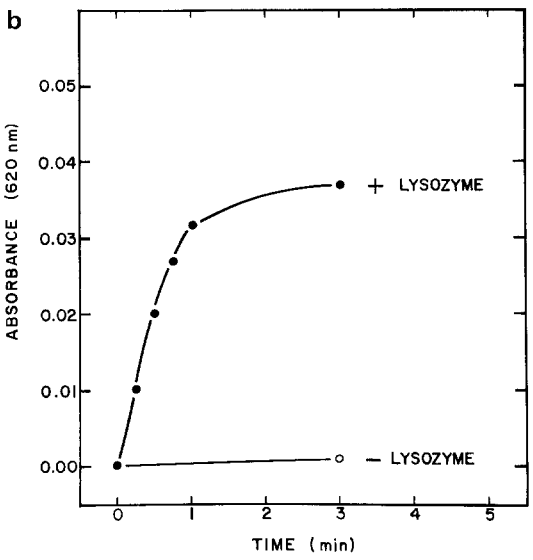
Morphology of the cyanelles in Glaucocystis

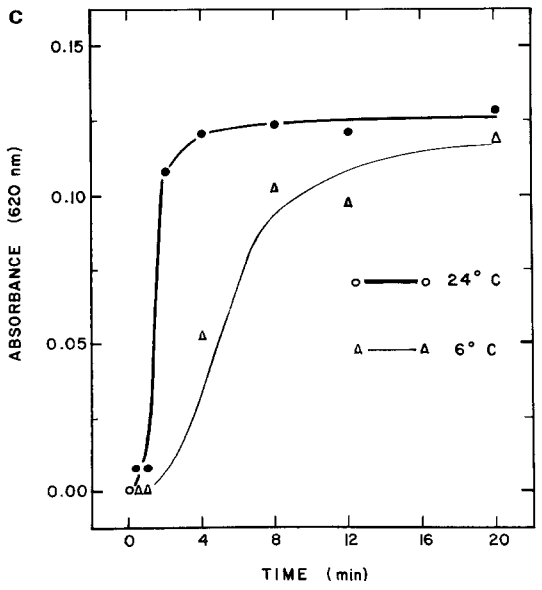
Ultrastructurally, the cyanelles resemble free-living cyanobacteria by the concentric arrangement of their photosynthetic lamellae and associated phycobilisomes, central nucleoplasm and ribosomes. In addition, several investigators have noted the presence of a less electron-dense, crenulating "outer" membrane surrounding the cyanelles (Fig. 1 b). Finally, the localization of a crystalline substance in what will eventually become the lamellar-free end can be observed in a small, immature cyanelle (Fig. 1 c).

Morphology of isolated cyanelles

Upon homogenization of *Glaucocystis* cells, "club-shaped" cyanelles (Fig. 2 a) are easily isolated in a hypotonic medium.





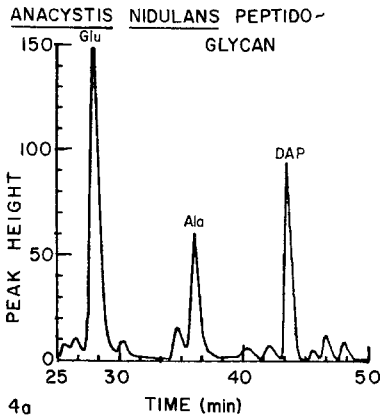


This shape is due to one or more swellings along the longitudinal axis of the cyanelle. Under Nomarski optics the tip of the narrower end of individual cyanelles appears lighter due to the absence of photosynthetic lamellae in this region.

Table 1. Influence of NAG-2 on the release of phycocyanin from isolated cyanelles by lysozyme (5 μg/ml) at 24° C

	Concentration of NAG-2 (mM)			
	0	2	10	30
Absorbance (620 nm)	0.047	0.040	0.030	0.018
Percent inhibition	—	7	35	62

Lysozyme treatment was for 2.5 min



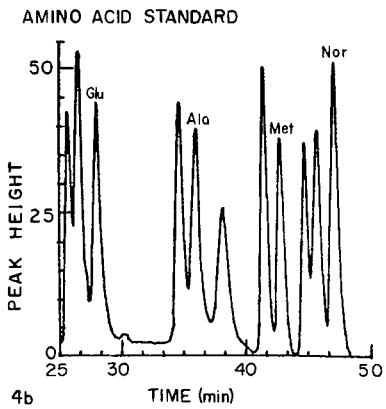


Fig. 4a, b. Amino acid analysis of peptidoglycan from *Anacystis nidulans*. **a** *Anacystis nidulans* peptidoglycan. Elution time (min): 28, Glu; 36, Ala; 44, DAP. **b** Amino acid standard (5 nM of each). Elution time (min): 26, Thr; 27, Ser; 28, Glu; 30, Pro; 35, Gly; 36, Ala; 38, Cys; 42, Val; 43, Met; 45, Ile; 46, Leu; 48, Nor (internal standard)

Fig. 3a–c. Lysis of *Glaucocystis* cyanelles by lysozyme. **a** Effect of lysozyme concentration on the release of phycocyanin in *Glaucocystis* cyanelles at 24° C. Treatment time was 2 min. **b**Time course of release of phycocyanin from isolated *Glaucocystis* cyanelles treated with lysozyme (5 μg/ml). **c** Effect of temperature on phycocyanin release from isolated *Glaucocystis* cyanelles treated with 5 μg/ml lysozyme

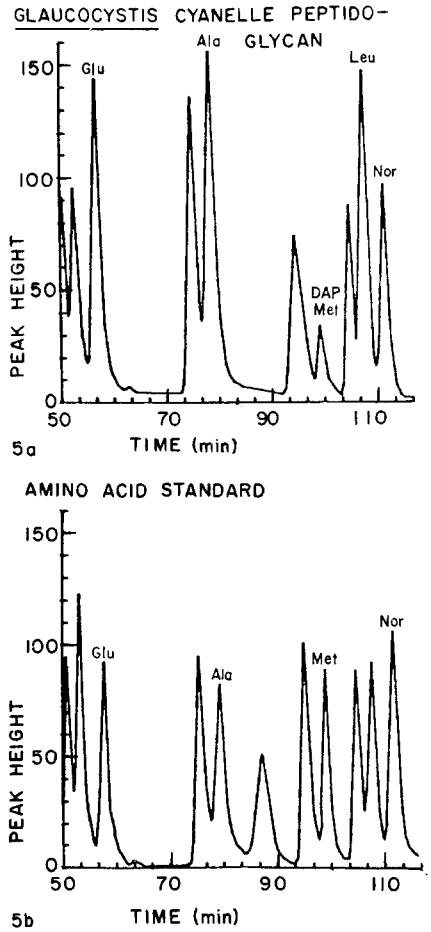


Fig. 5a, b. Amino acid analysis of partially purified (non-chromatographed) peptidoglycan from the cyanelles of *Glaucocystis nostochinearum*. **a** *Glaucocystis* cyanelle peptidoglycan. Elution time (min): 50, Thr; 53, Ser; 57, Glu; 63, Pro; 75, Gly; 78, Ala; 95, Val; 100, DAP and/or Met; 105, Ile; 107, Leu; 112, Nor. **b** Amino acid standard. Elution time (min): 51, Thr; 53, Ser; 58, Glu; 63, Pro; 75, Gly; 80, Ala; 87, Cys; 96, Val; 99, Met; 104, Ile; 108, Leu; 112, Nor

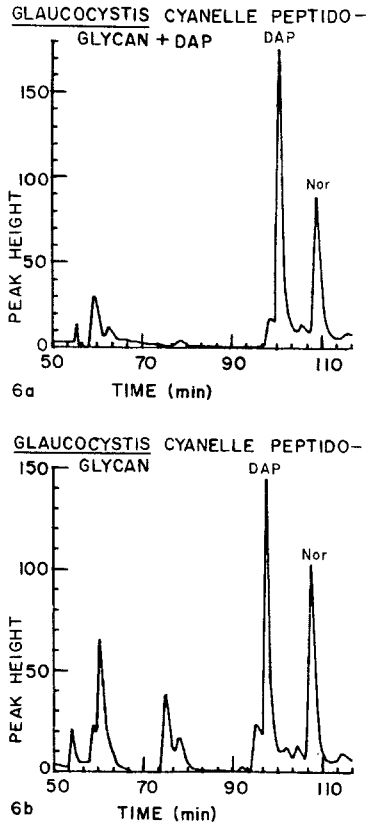


Fig. 6a, b. Amino acid analysis of chromatographed peptidoglycan from the cyanelles of *Glaucocystis*. **a** *Glaucocystis* cyanelle peptidoglycan. Elution time (min): 55, Thr; 60, Ser; 62, Glu; 101, DAP; 109, Nor (internal standard, 5 nM). **b** *Glaucocystis* cyanelle peptidoglycan + authentic DAP. Elution time (min): 98, DAP; 108, Nor (internal standard, 5 nM)

Lysozyme-sensitivity

The gross morphology of isolated *Glaucocystis* cyanelles is markedly affected by lysozyme. In hypotonic medium, the lysozyme-treated cyanelles rapidly swell and burst. However, if the medium is approximately isotonic, the cyanelles round up to form spheroplasts (Fig. 2b). Low concentrations of lysozyme are very effective in causing lysis, but no phycocyanin was released at higher concentrations (Fig. 3a). The optimum concentration was 5 µg of lysozyme per ml. Lysis of isolated cyanelles at this concentration (5 µg/ml) occurs very rapidly at 24°C and is essentially complete within 2 min (Fig. 3b).

In an effort to confirm that lysis is due to the enzymic activity of lysozyme, it was heat-treated for 15 min at 80°C. After this treatment, there was no lytic activity. In addition, reduction of the incubation temperature to 6°C retarded lysis (Fig. 3c).

Competitive inhibition of lysozyme activity by small oligomers of NAG

N-Acetyl-glucosamine (NAG), known to be a weak competitive inhibitor of lysozyme activity, caused a 44% inhibition at 0.5 M when assayed by the phycocyanin-release method. NAG-2, the dimer of NAG previously shown to be a much better inhibitor (Jollés et al. 1975; Sharon 1967), caused a 62% inhibition at a much lower concentration of 30 mM (Table 1).

Diaminopimelic acid analysis

The peptidoglycan from *Anacystis nidulans*, a Type A peptidoglycan (Drews 1973), was subjected to amino acid analysis for confirmation of the major amino acids which had been previously determined (Ghuysen 1968) to be present in a molar ratio of 2:1:1, alanine:glutamic acid:DAP, respectively. As indicated in Fig. 4a, the major peaks did conform with previous findings and, as such, provided a reliable method for determining the presence of DAP in the envelope surrounding *Glaucocystis* cyanelles.

Authentic DAP eluted at about the same time as Met (Fig. 4). The initial amino acid analysis of peptidoglycan from *Glaucocystis* cyanelles (Fig. 5) showed that the preparation was impure since more peaks were found than in the *Anacystis* peptidoglycan (Fig. 4a) and since the DAP and/or Met peak was relatively small in comparison to several other amino acids (Fig. 5).

The amino acid analysis of cyanellar peptidoglycan after paper chromatographic separation (Fig. 6a) was conducted at a higher sensitivity (due to the elimination of the major amino acids seen in Fig. 5). Finally, when authentic

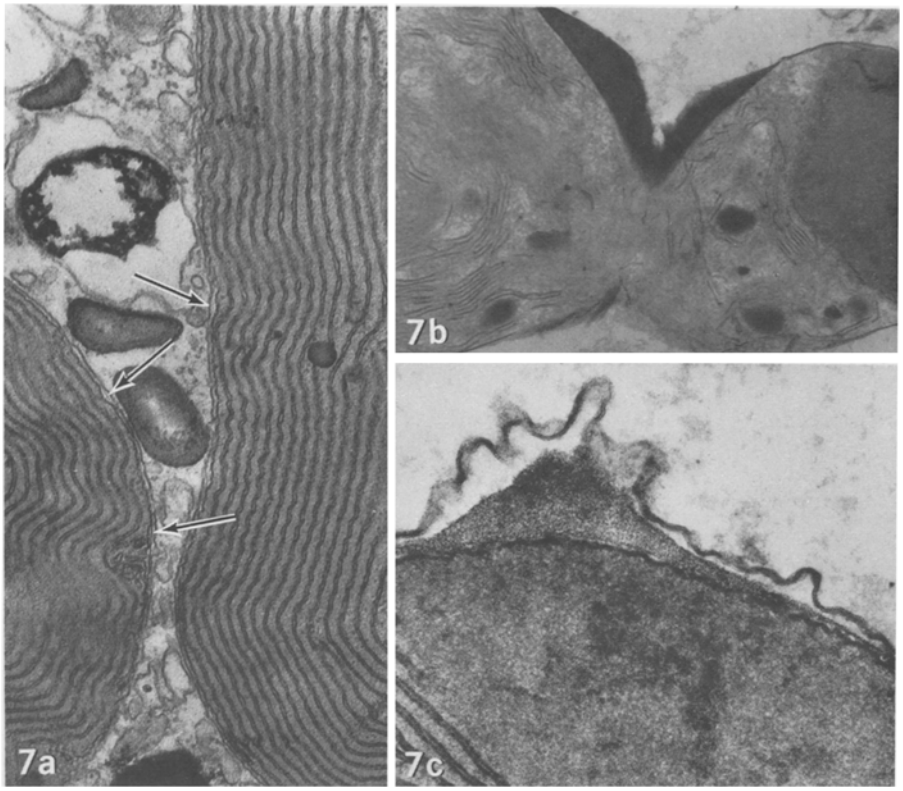


Fig. 7a–c
Ultrastructure of the cyanelle envelope. **a** Peptidoglycan in intracellular cyanelles at arrows, ×67,000. **b** "Wall substance" associated with division cleavage of a cyanelle in a *Glaucocystis* cell from a stationary culture, ×47,000. **c** "Wall substance" in an intracellular cyanelle appearing continuous with electron-dense material interpreted as peptidoglycan, ×115,000

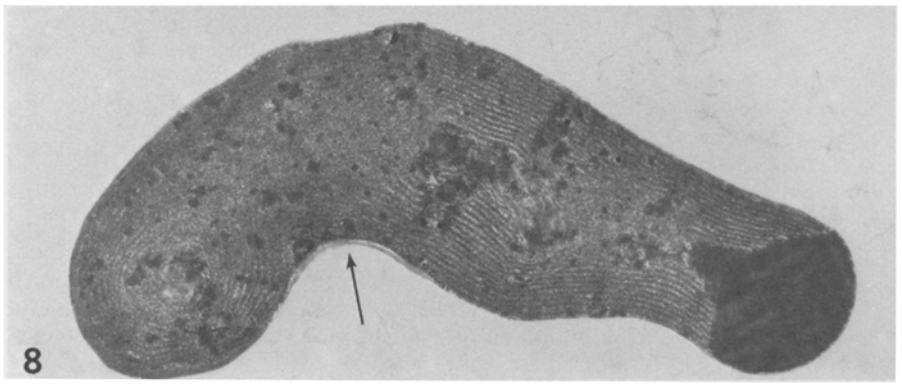


Fig. 8
Cyanelle isolated from *Glaucocystis* cell. "Wall substance" is visible at the arrow. The crenulating outer membrane is absent from isolated cyanelles, ×28,000

DAP (5 nM) was added to a duplicate of the sample illustrated in Fig. 6a, the DAP peak was enhanced relative to Nor, demonstrating that the peak at about 100 min represented DAP.

Ultrastructural analysis of the peptidoglycan envelope in the cyanelles

The peptidoglycan envelope in *Glaucocystis* cyanelles was not discernible in electron micrographs of all intracellular cyanelles. Only occasionally was a thin, rigid electron-dense structure observable between the limiting membrane of the cyanelle and the crenulating "outer" membrane (Fig. 1c). In many instances, the peptidoglycan envelope appears to be less electron dense and somewhat thinner than the cyanelle-limiting membrane (Fig. 7a). In addition, a "wall substance", usually associated with the division of a cyanelle, could be observed between the two membranes (Figs. 7b, c).

Finally, the ultrastructural examination of isolated cyanelles did not reveal the presence of the peptidoglycan envelope or the outer crenulating membrane in any of the sections examined (Fig. 8). Occasionally, however, the extracyanellar "wall substance", illustrated in Figs. 7b and c, was observed (Fig. 8).

Discussion

The cryptic nature of the phylogeny of *Glaucocystis* was last addressed in a freeze-fracture analysis of the host plasmalemma by Robinson and Preston (1971b). They suggested that the cyanelles of *Glaucocystis* could be normal plastids in one of the following algal types: (1) red alga, (2) primitive dinoflagellate, or (3) member of an as yet undescribed group. Earlier, other investigators (Geitler 1923, Pascher 1914; Margulis 1970, Stanier 1970) proposed an endosymbiotic origin for several phycological taxonomic enigmas, including *Glaucocystis*.

Schenk's demonstration in 1970 of a peptidoglycan envelope in *Cyanophora* cyanelles, helped certify that organism's

characterization as an endosymbiotic association and provided an impetus for subsequent studies (e.g., Herdman and Stanier 1977; Aitken and Stanier 1979; Trench et al. 1978; Floener and Bothe 1982; Giddings et al. 1983). The biochemical information currently available on endosymbiosis involving photosynthetic prokaryotes is, as a result, most extensive for *Cyanophora*. Necessarily, much of the future elucidation of biochemical aspects of endosymbiosis in *Glaucocystis* will be founded on the recent findings in *Cyanophora*.

Hall and Claus (1967) proposed that the name *Skujapelta nuda* be applied to the cyanelles from *Glaucocystis*. They were thought to be former cyanobacteria now existing as endosymbionts because they are "naked cells (which) are extremely sensitive to osmotic variations". The present report does not substantiate this finding, but instead offers the information that *Glaucocystis* cyanelles can be isolated readily from the intracellular environment without the protection of an osmoticum. Furthermore, the highly distinct morphology of the cyanelles is preserved and, as such, serves as indirect evidence for the presence of a cyanellar wall. Moreover, sensitivity of isolated cyanelles to lysozyme is consistent with the presence of a peptidoglycan envelope.

The standard concentration of lysozyme used by researchers to achieve peptidoglycan digestion in free-living cyanobacteria is 400 μg/ml (Jensen and Sicko 1971). Schenk (1970) used lysozyme at this concentration for his demonstration of lysozyme sensitivity in the cyanelles of *Cyanophora*. The same experimental protocol was unsuccessful with *Glaucocystis* (Schenk 1971). A basic finding of the present investigation was that the optimal lysozyme concentration for achieving lysis in isolated cyanelles from *Glaucocystis* was much lower (5 μg/ml). Lysozyme sensitivity of these cyanelles, as demonstrated by phycocyanin release, was not evident at lysozyme concentrations above 100 μg/ml (Fig. 3a). At high concentrations the cyanelle outlines became indistinct, but they did not swell or lyse. Others have reported that lysis of bacteria at enzyme concentrations above 20 μg/ml is slower (see Imoto et al. 1972).

Because *Cyanophora* does not possess a cell wall, Aitken and Stanier (1979) were able to isolate cyanelles by osmotic disruption of the host cell. Contrary to the view that *Cyanophora* and *Glaucocystis* represent similar endosymbiotic associations (Trench et al. 1978), the host cell in *Glaucocystis* is characterized by a thick cell wall consisting of a lamellate arrangement of cellulose microfibrils similar to the arrangement of microfibrils in secondary walls of higher plant cells (Robinson and Preston 1971a). This cell wall property enabled the host cell in *Glaucocystis* to be identified as a plant cell. Although host cells are clearly taxonomically distinct, the cyanelles share several traits. Besides their pigment composition, both possess "wall substance" (Pickett-Heaps 1972; Trench et al. 1978; Kies 1979) which, in *Glaucocystis*, has been observed in both the in vivo and in vitro states (Figs. 7b, 7c, and 8). This "wall substance" is clearly an extracyanellar component. Although it appears to be associated with the peptidoglycan layer (Fig. 7c), it does not have the staining properties of cyanobacterial peptidoglycan when visualized in the transmission electron microscope.

The present study demonstrates that cyanelles of *Glaucocystis* possess a peptidoglycan envelope. Supporting data include lysozyme sensitivity, evidence of competitive inhibition of the hydrolytic function of lysozyme, diminution of lysozyme activity at a lowered incubation temperature (6° C), ultrastructural visualization, and the positive demonstration of DAP in purified cyanellar envelopes. In the case of *Cyanophora*, the demonstration of the presence of DAP provided direct proof for the existence of an endosymbiotic association (Aitken and Stanier 1979). In the same manner, the present study provides the first unequivocal demonstration that *Glaucocystis nostochinearum* is an endosymbiosis in which the cyanelle has an envelope of the composition found in, and unique to, non-Archaebacterial prokaryotes. Therefore, the cyanelle in *Glaucocystis* should be viewed as a photosynthetic prokaryote which has become adapted to the intracellular environment of a taxonomically-uncertain eukaryotic algal host.

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