The Chemical Composition of Isolated Cell Walls of *Cyanidium caldarium*

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(Accepted for publication 9 July 1968)

**SUMMARY**

The acid-resistant and heat-resistant alga *Cyanidium caldarium* yields cell-wall preparations which are unusually rich in protein (50 to 55%) and contain only small amounts of polysaccharides (hemicellulose, 12 to 14%; cellulose, 3 to 4%). At least 13 amino acids are present in the cell walls, but diaminopimelic acid, muramic acid and amino sugars are absent. It is suggested that *Cyanidium* is more closely related to the green rather than the blue-green algae.

**INTRODUCTION**

The remarkably acid-tolerant and heat-tolerant unicellular alga *Cyanidium caldarium*, which will grow at pH 2.0 in sulphuric acid and at temperatures up to 80°, is a regular member of the microflora in acid hot springs throughout the world. Its taxonomic position is still undetermined and it possesses characteristics of blue-green as well as of green algae. The mode of reproduction by the formation of auto-spores is similar to that of the green alga *Chlorella* (Allen, 1959) and all of the organelles, except the pyrenoid, found in *Chlorella* can also be detected in *Cyanidium* cells (Rosen & Siegesmund, 1961; Mercer, Bogorad & Mullens, 1963; Staehelin, 1968). In contrast, in *Cyanidium* the chromoprotein C-phycocyanin is spectroscopically indistinguishable from that of the blue-green alga *Microcoleus rupicola* and, of the chlorophylls, only chlorophyll-a is present (Allen, 1959). Staehelin (1968) was able to show with the freeze-etching technique that *Cyanidium* cells, during their life cycle, also develop unusual surface differentiations which are not found on the plasmalemma membranes of either *Chlorella* or blue-green algae. Apart from a reported absence of diaminopimelic acid (Work & Dewey, 1953), the composition of *Cyanidium* cell walls does not appear to have been investigated. The present work was undertaken to see whether determinations of cell-wall composition of *C. caldarium* would help to explain its systematic position, and its tolerance to acid and heat.
METHODS

Organism. The strain of *Cyanidium caldarium* isolated by Allen (1959) was obtained from the Cambridge algal collection (Botany School, Cambridge).

Cultures. The organism was grown in the inorganic medium (pH 2.8) of Allen (1959) with glucose (10 g./l.) added aseptically to the sterilized medium. Volumes (1 l.) of medium in 2 l. conical flasks were inoculated and incubated in an enclosed gyratory shaker (Gallenkamp Ltd.) at 45° and 200 rev./min. for 7 days under continuous illumination (100 W electric light, 18 in. above shaker top window) with occasional (2 to 3 times/day) gassing with CO₂. During this time a heavy growth of organism, initially yellowish and finally dark green, was obtained; the cultures reached finally pH 1.8 to 1.9. The organisms were allowed to settle under gravity and most of the liquid decanted; the more concentrated suspension was then centrifuged (1000 g) after which the deposited organisms were washed by suspension in water and then recentrifuged down.

Preparation of cell walls. Wet organisms, diluted to a thin paste with water, were mixed with an equal weight of Ballotini beads (no. 13) and disrupted by blending for $4 \times 10$ min. at full speed in a Lourdes Omnimix homogenizer with the blending-pot immersed in an ice bath. The blended mixture was diluted with water and the suspension of disrupted organisms decanted from the beads, which were then washed free from residual cell debris. Any undisrupted organisms and beads were removed from the combined suspensions by centrifugation at 1000 g for 2 to 15 min. after which the walls were harvested by centrifugation for 10 min. at 4000 g. The initial crude wall preparation was washed 3 to 4 times by suspension in water and centrifugation until the aqueous supernatant fluid was clear and colourless. Finally the grey wall preparation was washed by suspension for 5 min. and centrifugation, twice from m-NaCl and then twice from water to give preparation A. One portion of wet preparation A was washed by a similar treatment once in 0.5% sodium lauryl sulphate solution for 5 min. followed by two washings with water to give preparation B; a second portion of preparation A was similarly washed 4 times with the sodium lauryl sulphate solution and twice with water to give preparation C. All these wall preparations were freeze-dried.

Microscopical examination of the wall preparations. The wall preparations were examined by the optical and electron microscopes. Sectioned material for the electron microscope was prepared by fixing the walls in 2% KMnO₄ in tap water for 30 min., after which they were washed with water, dehydrated with ethanol and finally embedded in methacrylate (10 parts butyl-, 3 parts methyl-methacrylate, with 3% dichlorobenzoylperoxide (Luperco CDB; Lucidal Div., Novadel-Agene Corp., Buffalo, N.Y.) as accelerator) which was polymerized overnight at 60°. Unsectioned walls were shadowed with U₃O₈ after allowing a dilute suspension of walls to dry in air on Formvar-carbon-coated grids. Freeze-etch replicas for electron microscopy were also prepared by the technique of Moor & Mühlethaler (1963), but it was difficult to assess the purity of isolated wall fractions by this method.

Analyses of walls. Moisture and ash contents of the walls were measured by heating, respectively, at 100° and 500°.

Total nitrogen was measured by the micro-Kjeldahl procedure of Bathurst & Mitchell (1958). Total protein was measured by the method of Lowry, Rosebrough,
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Farr & Randall (1951) as modified for insoluble protein. Total crude lipids were measured by extracting the walls by shaking at room temperature with chloroform + methanol (3 + 1 by vol.) for several hours. The combined extracts were evaporated to dryness and the residue weighed.

Carbohydrates were analysed by a variety of methods. Water-soluble polysaccharide and pectic substances in the walls were extracted by successive treatments for 15 min. with boiling water (25 ml.) and for 2 hr with boiling ammonium oxalate (0·5%, 50 ml.). Polymer hexoses and uronic acids were measured with anthrone and carboxazole reagents, respectively (Bailey, 1967). Hemicelluloses and cellulose were measured by differential acid hydrolysis. Portions (20 mg.) of wall were hydrolysed for 2 hr. at 100° in N-H₂SO₄ and centrifuged to give a supernatant fluid (containing hemicellulose sugars) and a deposit which was dried with acetone. The deposit material was treated with 72% H₂SO₄ (0·2 ml.) for 4 hr at room temperature, diluted with water (4 ml.) and heated at 100° for 2 hr, to yield a second hydrolysate (containing cellulose sugars). Reducing sugars in the hydrolysates were measured by the microcuprimetric method of Nelson (1944) and calculated as anhydro-xylene and anhydro-glucose for hemicellulose and cellulose, respectively. Portions of the hydrolysates were neutralized with solid BaCO₃, filtered and concentrated for paper chromatographic analysis.

The nature of the possible hemicellulose and cellulose polymers was further examined by their solubilities in alkali. A portion (50 mg.) of wall was extracted by shaking overnight at room temperature with 2 x 10 ml. of aqueous KOH (24% w/v) and centrifuged, after which the alkali-insoluble residue was hydrolysed by treatment with 72% H₂SO₄. The alkaline solution was acidified with acetic acid and ethanol (2 vol.) added, to give a precipitate which was hydrolysed in N-H₂SO₄ for 2 hr at 100°. The acid hydrolysates were treated with BaCO₃ and prepared for paper chromatography as already described.

Chromatograms for monosaccharide analyses were developed with ethyl acetate + water + pyridine (2 + 2 + 1, by vol.) or ethyl acetate + acetic acid + formic acid + water (9 + 1·5 + 0·5 + 2, by vol.) and sugars located with aniline hydrogen phosphate spray reagent. Quantitative chromatograms used the method of Wilson (1959) and the same aniline hydrogen phosphate spray reagent when the papers were sprayed and heated under carefully controlled conditions which gave good recoveries (over-all error ± 5%) of sugars. Amino sugars in the amino acid hydrolysate were located on chromatograms developed either with the solvent systems used for sugar analysis, or with those used for amino acid analysis, with the following spray reagents: ninhydrin + collidine, p-dimethylaminobenzaldehyde (Partridge, 1948; Crumpton, 1959) and thiobarbiturate (Warren, 1960).

For amino acid analysis hydrolysates were prepared by heating the walls (20 mg.) at 100° for 18 hr in 6 N-HCl (2 ml.) in a sealed tube and then removing the acid by repeated evaporation in vacuum over solid NaOH. Amino acids were identified on chromatograms developed in phenol-water, followed by n-butanol + acetic acid + water (60 + 15 + 25, by vol.), and in ionophoretograms run in acetic acid + formic buffer (pH 2·0, Smith, 1960) and sprayed with ninhydrin + collidine.
RESULTS

The yield of wet organisms from 16 l. of culture was 266 g., containing 13.5% of freeze-dried solid. From 160 g. of these wet organisms (21.6 g. freeze-dried solid) 1.82 g. freeze-dried preparation A was obtained (1.64 g. corrected for moisture). All of the freeze-dried wall preparations contained about 10% moisture and all results given below are on a moisture-free basis.

According to Punnett & Derrenbacker (1966) prolonged washing with sodium lauryl sulphate is necessary to remove gross contamination of cytoplasmic protein from many algal wall preparations. When a portion of preparation A was submitted to this type of prolonged washing procedure to give preparation C, the total nitrogen removed in the combined washings (0.59 mg.) represented only 4.5% of the total nitrogen (12.9 mg.) in the initial preparation A, suggesting that only a small amount of preparation A had actually been solubilized.

Microscopic examination of wall preparations

Whole Cyanidium caldarium organisms are easily distinguished from isolated walls in the light microscope. In the wall preparations used for chemical analysis only very few intact organisms were detected. Micrographs of a sectioned permanganate-fixed pellet of preparation A (Pl. 1, fig. 1, 2) showed the walls to be strongly stained and revealed no layering, while the small amount of cytoplasmic debris still adhering to the walls showed as dark granular material (Pl. 1, fig. 2). A similarly prepared micrograph of preparation B (Pl. 1, fig. 3) showed that the single sodium lauryl sulphate washing had nearly completely removed the cytoplasmic contamination without affecting the walls. The micrographs of preparation C (Pl. 1, fig. 4), however, showed that the prolonged washing with sodium lauryl sulphate resulted in a partial disintegration of the walls into smaller fragments. After heavy-metal shadowing electron microscopy of air-dried wall preparation A showed the walls to have a fine granular structure (Pl. 1, fig. 5, 6). Several attempts to detect fibrils, by electron microscopy, after removing some of the matrix from the walls by various treatments, both separately and sequentially with potassium hydroxide (4%, w/v), hydrochloric acid (0.25 M) and concentrated hydrogen peroxide + glacial acetic acid mixture (1 + 1, by vol.) met with no success, probably because of the very small amount of cellulose present. In these attempts unstructured masses of sponge-like material were formed or the walls disintegrated completely. The absence of highly ordered material such as fibrils in the walls of Cyanidium was confirmed with X-ray diagrams of untreated isolated walls. The diagrams indicated the presence of a keratin-like substance, but the lines were always very weak and diffuse.

Composition of the walls of Cyanidium caldarium

Results from the analyses of the wall preparations are listed in Table 1. Qualitative and quantitative paper chromatograms of the hemicellulose hydrolysates from the analyses showed spots corresponding to galactose, glucose, mannose and xylose in the ratio of 5.2:4.5:2.5:1.0, but only traces of arabinose and uronic acids and no methylpentoses. The cellulose hydrolysates showed only glucose on the chromatograms. After prolonged extraction of a portion of wall preparation A with 24% KOH the alkali-insoluble residue gave a hydrolysate showing only glucose on the chromatograms.
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grams. Hydrolysates of the alkali-soluble polysaccharide showed on the chromatograms all of the expected hemicellulose sugars, with evidence, not further examined, that much of the galactose was present in a more soluble polymer than were the glucose, mannose and xylose.

Table 1. Composition of Cyanidium caldarium cell walls

All results are calculated as % of moisture-free cell walls.

<table>
<thead>
<tr>
<th>Wall preparation</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash</td>
<td>10.0</td>
<td></td>
<td>5.8</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>8.96</td>
<td>8.55</td>
<td>9.11</td>
</tr>
<tr>
<td>Total lipids</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hot-water-soluble polysaccharide</td>
<td>0.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxalate-soluble polyuronide (pectin)</td>
<td>0.035</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>13.73</td>
<td>15.00</td>
<td>14.50</td>
</tr>
<tr>
<td>Cellulose</td>
<td>3.83</td>
<td>2.74</td>
<td>4.11</td>
</tr>
</tbody>
</table>

Table 2. Amino-acid composition of Cyanidium caldarium cell walls

Amino acids detected with ninhydrin + collidine on paper chromatograms of 6 N HCl hydrolysates. Intensities of spots judged visually and graded as very intense, ++++; moderately intense, +++; weak intensity but definite, ++; faint, +; absent, -.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Wall preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>++++</td>
</tr>
<tr>
<td>Serine</td>
<td>++++</td>
</tr>
<tr>
<td>Threonine</td>
<td>++++</td>
</tr>
<tr>
<td>Alanine</td>
<td>++++</td>
</tr>
<tr>
<td>Glycine</td>
<td>++++</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>++++</td>
</tr>
<tr>
<td>Valine</td>
<td>++++</td>
</tr>
<tr>
<td>Leucine-isoleucine</td>
<td>++</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>++</td>
</tr>
<tr>
<td>Proline</td>
<td>++</td>
</tr>
<tr>
<td>Lysine</td>
<td>++</td>
</tr>
<tr>
<td>Methionine</td>
<td>+</td>
</tr>
<tr>
<td>Histidine</td>
<td>+</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>+</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>+</td>
</tr>
<tr>
<td>Arginine</td>
<td>+</td>
</tr>
<tr>
<td>Unknown blue spots</td>
<td>X1</td>
</tr>
<tr>
<td></td>
<td>++++</td>
</tr>
</tbody>
</table>

The amino acid composition of the three wall preparations is listed in Table 2. Cysteine and diaminopimelic acid were not detected in the hydrolysates. Unknown X1, present in significant amounts, moved on the chromatograms in almost the same way as diaminopimelic acid, although its stain colour with collidine + ninhydrin was slightly different. Ionophoresis at pH 2.0 of wall hydrolysate with and without added authentic \( \alpha,\epsilon\)-diaminopimelic acid clearly showed none of this acid in the hydrolysates and that the unknown X1 was different. There was no sign of components...
corresponding to glucosamine, galactosamine or muramic acid on chromatograms of any of the wall preparation hydrolysates.

The wall preparations could not be dissolved in n-alkali in sufficient concentration for the biuret measurement of total protein. With the Folin reagent an alkaline solution gave an intense blue colour, corresponding in the case of preparation C to 65% protein (bovine serum albumin as standard).

DISCUSSION

The wall carbohydrates of several green algae have been examined and a mixture of polysaccharides composed principally of galactose, mannose, xylose and glucose appears to be fairly common (Kreger, 1962). Northcote, Goulding & Horne (1958) found that the walls of Chlorella were rich in hemicellulose polymers (30%) composed of galactose, glucose, mannose, xylose and arabinose, and cellulose (16%). Although a mixture of carbohydrates composed of the same monosaccharides was found in the Cyanidium walls the amounts were very much lower and, in the case of cellulose, very low indeed. The walls of fewer examples of blue-green algae have been examined chemically (Echlin & Morris, 1965), and these were found to contain mucoprotein with amino sugar and muramic acid rather than simple polysaccharides. Neither glucosamine nor muramic acid was found in the Cyanidium cell wall hydrolysates. Northcote et al. (1958) and Punnett & Derrenbacker (1966) detected glucosamine in Chlorella walls.

The presence of a large variety of amino acids in the wall protein component of Cyanidium is in line with findings for Chlorella (Northcote et al. 1958), Nitella and Chaetomorpha (Thompson & Preston, 1967) and higher plants (Lamport, 1965). Diaminopimelic acid, typically present in bacterial and blue-green algal walls (Echlin & Morris, 1965), was clearly absent from the wall hydrolysates of Cyanidium, a finding in agreement with the results of Work & Dewey (1953) for whole Cyanidium organisms.

The present results tend to support the suggestion that Cyanidium caldarium occupies an intermediate position between the blue-green and the green algae, with possibly a closer relationship to the green than to the blue-green algae.

The most outstanding feature to emerge from the present work with Cyanidium caldarium walls is the high (50 to 55%) protein content. There is of course the possibility that some of this protein is cytoplasmic protein which has become bound to the cell walls during their isolation. This seems unlikely in view of the limited spectrum of amino acids found in the hydrolysates and the evidence for the removal of cytoplasmic debris by washing (Table 1 and Pl. 1, fig. 2, 3). As far as we know, no other plant or algal walls have been reported with such a high percentage of protein. Typical results are Chlorella pyrenoidosa, 7 to 9% (Northcote et al. 1958); Nitella, 8 to 10%, Chaetomorpha, 7 to 9% (Thompson & Preston, 1967); and Sycamore cell cultures, 7.5 to 11% (Lamport, 1965). All electron microscope preparations of the Cyanidium walls indicate that the material is very dense and it seems possible that the closely packed proteins could provide the cells with more than just mechanical protection. Perhaps the high protein content of the walls is related to the heat and acid tolerance of the organisms.
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Thanks are due to Mrs V. Pain and Mr I. Manning for analytical assistance and to Dr P. J. Peterson for help in amino acid analyses.

REFERENCES


EXPLANATION OF PLATE

Fig. 1. Section of isolated walls of *Cyanidium caldarium* washed with water and m-NaCl (preparation A), fixed with 2% KMnO₄ and embedded in methacrylate. × 16,000.

Fig. 2. Higher magnification of the same preparation as in fig. 1. The granular material adhering to the cell wall surfaces is probably due to cytoplasmic contamination. × 30,000.

Fig. 3. Sectioned walls prepared as described for fig. 1 but additionally washed with 0.5% Na laurylsulphate for 5 min. (preparation B). This treatment removed nearly all the granular material from the wall surfaces. × 30,000.

Fig. 4. Sectioned walls prepared as in fig. 1 but treated additionally for 4 × 5 min. with 0.5% Na laurylsulphate (preparation C). The walls show signs of disintegration. × 30,000.

Fig. 5. Isolated wall of *C. caldarium* washed with water and m-NaCl (preparation A) and shadowed with U₃O₈. × 22,000.

Fig. 6. Higher magnification of the same preparation as Fig. 5. The wall material appears finely granular; no fibrils detectable. × 40,000.