Basis for the Resistance of Several Algae to Microbial Decomposition

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The basis for the resistance of certain algae to microbial decomposition in natural waters was investigated using *Pediastrum duplex*, *Staurastrum* sp., and *Fischerella muscicola* as test organisms. Enzyme preparations previously found to convert susceptible algae into spheroplasts had no such effect on the resistant species, although glucose and galacturonic acid were released from *P. duplex* walls. Little protein or lipid but considerable carbohydrate was found in the walls of the refractory organisms, but resistance was not correlated with the presence of a unique sugar monomer. A substance present in *Staurastrum* sp. walls was characterized as lignin or lignin-like on the basis of its extraction characteristics, infrared spectrum, pyrolysis pattern, and content of an aromatic building block. Sporopollenin was found in *P. duplex*, and cellulose in *Staurastrum* sp. Cell walls of the algae were fractionated, and the fractions least susceptible to microbial degradation were the sporopollenin of *P. duplex*, the polyaromatic component of *Staurastrum* sp., and two *F. muscicola* fractions containing several sugar monomers. The sporopollenin content of *P. duplex*, the content of lignin or a related constituent of *Staurastrum* sp., and the resistance of the algae to microbial attack increased with age. It is suggested that resistance results from the presence of sporopollenin in *P. duplex*, a lignin-like material in *Staurastrum* sp., and possibly heteropolysaccharides in *F. muscicola*.

The cell wall probably is a major determinant of the resistance or susceptibility of algae to microbial decomposition in natural ecosystems inasmuch as it represents a key obstructing structure which must be disrupted, either mechanically or enzymatically, before an attacking organism has access to the contents of the algal protoplast and causes death of the organism. Although considerable work has been done to determine which components of the cell walls of fungi render these organisms refractory to microbial decomposition (3, 5), little is known about the components of algal cell walls which protect them from possible attack in nature.

In a study of the susceptibility of several algae to microbial degradation in natural waters, it was observed that *Staurastrum* sp., *Fischerella muscicola*, and *Pediastrum duplex* were particularly resistant to attack under conditions where other algae were readily destroyed and their contents liberated (Gunnison and Alexander, Limnol. Oceanogr., in press). The present investigation was initiated to determine which components in the cell walls were responsible for the resistance to microbial degradation.

**MATERIALS AND METHODS**

*Staurastrum* sp., a planktonic freshwater alga, was obtained from the Culture Collection of Algae at Indiana University. *F. muscicola*, a cosmopolitan blue-green alga (19, 22), was from Carolina Biological Supply Co., Burlington, N. C. *P. duplex*, another planktonic freshwater green alga, came from the Cambridge University Culture Collection of Algae and Protozoa. The methods used to grow and harvest these microscopic algae and to determine their resistance to microbial decomposition are described elsewhere (Gunnison and Alexander, Can. J. Microbiol., in press). To prepare the walls and wall fragments, the algae were lyophilized, suspended in cold (4°C) 0.01 M phosphate buffer (pH 7.0), and then ruptured by use of a sonic oscillator (Branson Sonic Power Co., Plainview, N. Y.) until 95 to 100% of the cells appeared broken when viewed under the microscope. The preparations were washed several times with the buffer until cellular material was no longer found in the supernatant fluid; the walls were recovered after each washing by centrifugation at 270 × g for 20 min. After treatment with 1.0% sodium dodecyl sulfate, the wall preparations were washed in distilled water and then lyophilized. The sensitivity of the walls to enzymatic hydrolysis and the carbohydrate, protein,
aerial acid, lipid, uronic acid, and sugar content of the walls were determined in the same manner as described for susceptible walls (Gunnison and Alexander, Can. J. Microbiol., in press).

The walls were fractionated by a procedure modified from that of Boroughs and Epstein (6). A 50-mg sample of walls was treated for 2 h with 20 ml of 0.2% ammonium oxalate at room temperature, and the residue (designated I-1) was removed by centrifugation. The soluble fraction was designated S-1. Fraction I-1 was washed with distilled water and then treated with 20 ml of 0.05 N HCl at 85 °C for 4 h. The insoluble residue was removed by centrifugation and discarded, the solubilized material being designated fraction S-2. A separate 100-mg portion of walls was incubated for 4 h at 4 °C in 20 ml of 4% NaOH, and the soluble fraction (S-3) was separated from the unextracted material (fraction F-2) by centrifugation. Fraction F-2 was then suspended for 4 h in 17.5% NaOH at 4 °C, and the suspension was separated into a soluble (S-4) and an insoluble fraction (I-3) by centrifugation. Fractions S-1, S-2, S-3, S-4, and I-3 are stated to contain pectates, protopectin, polyuronide hemicelluloses, noncellulosic polysaccharides, and α-cellulose, respectively (6). All materials were extracted twice in 20-ml volumes of extraction solution. Each insoluble residue was collected by centrifugation at 31,000 x g and washed once in 20 ml of distilled water. The two extracts and the water used to wash the insoluble residue derived from a given extracting solution were pooled.

The extracts were taken to dryness in a rotary evaporator (Rinco Rotavapor). Fraction S-1 was then dissolved in 8.0 ml of distilled water. Prior to concentration, fractions S-2, S-3, and S-4 were neutralized, and after concentration, they were suspended in distilled water, dialyzed against distilled water at 4 °C for 12 h, reconstituted, and then again suspended in distilled water. Fraction I-3 was washed 10 times in distilled water and then lyophilized.

For analysis of the fractions, either 10 ml of a soluble fraction or 10 to 15 mg of fraction I-3 was hydrolyzed in a sealed ampoule for 12 h with 2 ml of 6 N HCl at 105 °C. After removal of excess HCl at 50 °C under vacuum, the hydrolyzate was examined for total carbohydrate, reducing sugars, and sugar monomers, the latter by thin-layer chromatography (Gunnison and Alexander, Can. J. Microbiol., in press). Protein content was assessed after hydrolysis of the fraction for 60 min with 1 N NaOH at room temperature followed by neutralization with HCl; protein was determined by the Folin phenol method (13).

A lignin-like material was extracted from Staurastrum sp. walls and from pine wood sawdust by the H₂SO₄ extraction method of the Technical Association of Pulp and Paper Industries (18). Sporopollenin was prepared from P. duplex by the sequential extraction method of Zetsche and Vicari (23). For infrared analysis, 2 to 4 mg of the material was made into a pellet with 40 mg of KBr, and the spectra were obtained with an infrared spectrophotometer, model IR-10 (Beckman Instruments, Fullerton, Calif.). The quantity of apparent lignin and sporopollenin in the wall preparations was established by determining the residue remaining after the extraction.

For pyrolysis-gas liquid chromatographic analysis, 100 µg of the lignin preparation was applied to a platinum pyrolysis coil; the latter was subsequently fired at 800 °C in a model A-25 Pyrolyzer Accessory (Wilkens Instruments, Walnut Creek, Calif.). Separation of the resulting products was accomplished by gas chromatography using a stainless-steel column (3 mm diam by 2 m long) packed with Chromosorb W, 60 to 80 mesh (Johns Mansville, New York), as a solid support for the SE-52 liquid phase (Applied Science Laboratories, State College, Pa.). The products of pyrolysis were carried onto the column with N₂ flowing at a rate of 20 ml/min. The column was held at 50 °C for the first 6 min after pyrolyzer firing, and it was then subjected to a programmed temperature rise of 8.6 °C/min until 185 °C was reached. A flame ionization detector was used to monitor the exit of products from the column.

Analysis of the fused lignin for phenylcarboxylic acids by thin-layer chromatography was performed on precoated plates of silica gel containing a fluorescent indicator (Eastman Kodak Company, Rochester, N.Y.) using benzene-methanol-acetic acid (90:16:8) as the solvent system (17). The solution of fused lignin was analyzed for catechols by gas chromatography using a modification of the procedure of Helling and Bollag (10) in which 10% DC 200 (Applied Science Laboratories) on Chromosorb W, 80 to 100 mesh (Johns Mansville), was used as the stationary phase. For gas chromatography, the injector, column, and detector temperatures were 260, 230, and 290 °C, respectively. The flow rate of the carrier gas, N₂, was 60 ml/min.

To obtain ¹³C-labeled walls, the cultures were harvested after 14 days, and the algal cells collected from 8 liters of medium were placed in each of two 2-liter Erlenmeyer flasks. To these flasks was added 1.5 liters of the culture supernatant which had been adjusted to pH 8.3, and 0.5 ml of NaH¹³CO₃ (New England Nuclear, Boston, Mass.) having an activity of 1 mCi/ml was then introduced. The algal suspensions were incubated on a rotary shaker at 150 rpm at 22 °C under light at 5,400-lux intensity, and the labeled cells were harvested after 7 days, washed in 0.01 M phosphate buffer (pH 7.0), and lyophilized. Cell walls and wall fractions were prepared as described above.

To determine the resistance of labeled walls and wall fractions to microbial attack, 50 mg of walls, 10 to 90 mg of a wall fraction, 21 mg of lignin, or 18 mg of sporopollenin was suspended in distilled water to a final volume of 10 ml. To each of two 250-ml Erlenmeyer flasks were added 5.0 ml of a suspension of the walls or an isolated fraction, 35 ml of the salts solution, and 0.25 g of soil (Williamson silt loam) contained in 2.5 ml of distilled water. In some instances, 2 µCi of ¹³C glucose rather than walls or a wall fraction was used as the sole carbon source. The flasks were incubated at 25 °C on a shaker operating at 120 strokes/min. Samples were taken at regular intervals, and the loss rate of labeled carbon was determined by a modification of the method of Hobbie and
plast formation with susceptible species (Gunnison and Alexander, Can. J. Microbiol., in press), did not result in the conversion of Staurastrum sp. or P. duplex cells into spheroplasts.

**Composition of the walls.** The amino acid content of walls of two of the algae is given in Table 2. No amino acid was dominant, and the quantitative composition differed among the two species. It is interesting that the results for the green alga, P. duplex, were quite similar qualitatively and quantitatively to those found in Cylindropermum sp. (Gunnison and Alexander, Can. J. Microbiol., in press), a blue-green alga that is decomposed readily by microorganisms. Amino acids were not detected in Staurastrum sp. cell walls, but it is possible that levels below the sensitivity of the ninhydrin method used (15) were present.

The protein, carbohydrate, uronic acid, and lipid composition of the cell walls is presented in Table 3. The wall of Staurastrum sp. is dominated by carbohydrate and uronic acids, and no lipids were found. Carbohydrates are also prominent in the walls of F. muscicola and P. duplex, but their abundance is not as great as in Staurastrum sp. The data for carbohydrate and uronic acid content of F. muscicola are anomalous because of the high recoveries, and it is likely that interfering substances affected the analytical determinations.

A comparison of the sugar and uronic acid monomers found in acid hydrolyzates of the cell walls is presented in Table 3. Glucose was abundant in the three organisms, but different

| Amino acid content as % of wall | Fischereilla muscicola | Pediastrum duplex |
|-------------------------------|-----------------------|-------------------|
| Alanine                       | 0.6                   | 1.7               |
| Asparagine                    | 0.0                   | 0.5               |
| Aspartic acid                 | 0.7                   | 1.4               |
| Cystine                       | 0.0                   | 0.8               |
| Glutamic acid                 | 0.7                   | 1.3               |
| Glutamine                     | 0.7                   | 0.0               |
| Glycine                       | 0.8                   | 1.5               |
| Isoleucine                    | 0.4                   | 0.5               |
| Leucine                       | 0.3                   | 1.1               |
| Lysine                        | 0.0                   | 1.1               |
| Methionine                    | 0.0                   | 0.0               |
| Phenylalanine                 | 0.0                   | 0.7               |
| Proline                       | 0.0                   | 0.8               |
| Serine                        | 0.5                   | 1.9               |
| Threonine                     | 0.3                   | 1.2               |
| Tyrosine                      | 0.0                   | 0.4               |
| Valine                        | 0.3                   | 1.2               |

**Table 2. Amino acid composition of algal cell walls**

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Crawford (11). For this purpose, labeled \( \text{CO}_2 \) that remained in the reaction flask at the sampling time was driven off by acidification with 0.5 ml of 2 N HCl, and 0.50-ml amounts of the acidified reaction mixture were placed in scintillation vials with 10 ml of scintillation solution (7). The radioactivity was then determined in a scintillation counter, model Mark II (Nuclear Chicago, Chicago, Ill.).

**RESULTS**

**Enzymatic digestion of cell walls.** The sources of the various enzyme preparations used have already been described (Gunnison and Alexander, Can. J. Microbiol., in press). Chitinase, \( \beta \)-glucuronidase, hemi-cellulase, lipase, \( \alpha \)-lysozyme, Pronase, and lytic enzyme preparations of *Streptomyces* isolates G4 and G7 had no effect on walls of these three resistant algae. Similarly, cellulase (free of polygalacturonase activity) was without effect on walls of *F. muscicola* and *Staurastrum* sp., and the polygalacturonase preparation did not digest *F. muscicola* walls. On the other hand, carbohydrate was released from the walls of *P. duplex* by cellulase and polygalacturonase preparations. Most of the solubilized carbohydrate appeared to be glucose, although the Bitter and Muir (4) test showed the presence of some galacturonic acid (Table 1). Essentially all of the wall material solubilized enzymatically could be accounted for as glucose and galacturonic acid. The polygalacturonase preparation had cellulase activity as determined by the formation of glucose from cellulose, but it released no sugar from \( \beta-1,2 \) glucan, \( \beta-1,6 \) glucan, laminarin, or starch. The polygalacturonase preparation also released small amounts of carbohydrate from *Staurastrum* sp., but neither reducing sugars nor specific products were found in the hydrolyzates. By contrast, incubation of these two enzyme preparations with intact algae, using the methods previously shown to lead to spheroplast formation with susceptible species (Gunnison and Alexander, Can. J. Microbiol., in press), did not result in the conversion of *Staurastrum* sp. or *P. duplex* cells into spheroplasts.

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**Table 1. Activity of cellulase and polygalacturonase preparations on algal cell walls**

| Enzyme prep | Source of wall preparation | % Carbohydrate solubilized | Soluble fraction (% of wall) | Reducing sugar | Glucose | Galacturonic acid |
|-------------|---------------------------|-----------------------------|-----------------------------|----------------|---------|------------------|
| Cellulase   | *Pediastrum duplex*       | 27                          | 24                          | 23             | 0       |                  |
| Polygalacturonase | *Pediastrum duplex*       | 32                          | 34                          | 31             | 3       |                  |
|             | *Staurastrum* sp.          | 4                           | 0                           | 0              | 0       |                  |

*Percent of cell wall converted to anthrone-positive materials.

*Percent of cell wall converted to reducing sugar, glucose, and uronic acid.
amounts of xylose and galacturonic acid were present in the algae. Only *Staurastrum* sp. contained galactose, but it had no fucose al-

**Table 3. Chemical composition of the walls of three algae**

| Wall component | *Staurastrum* sp. | Fischerella muscicola | Pediastram duplex |
|----------------|------------------|-----------------------|-------------------|
| **A**          |                  |                       |                   |
| Protein       | 0.0              | 5.3                   | 16.2              |
| Lipid         | 0.0              | 0.0                   | 0.7               |
| Carbohydrate  | 80               | 61                    | 48                |
| Uronic acids  | 20               | 77                    | 8.2               |
| **B**          |                  |                       |                   |
| Glucose       | + + +            | + + +                 | + + +             |
| Mannose       | + + + +          | + + + +               | + ++              |
| Xylose        | + + +            | + + + +               | + + +             |
| Fucose        | - + +            | - + +                 | +                 |
| Galactose     | + -              | -                     | -                 |
| Galacturonic acid | +       | +                    | + + +             |

*Results shown as: A, percentage of wall weight; B, relative abundance (based on comparison of spot sizes after thin-layer chromatography of hydrolyzates). By summation of values for individual amino acids.*

**Composition of wall fractions.** The values obtained from chemical analyses of the wall fractions are presented in Table 4. It is evident that only a small percentage of the *Staurastrum* sp. wall was solubilized by the ammonium oxalate, HCl, and NaOH treatments which were used to obtain fractions S-1, S-2, S-3, and S-4. Fraction I-3, which had residual wall constituents not solubilized by NaOH, contained 78% of the *Staurastrum* sp. material which was originally subjected to the alkali treatments, and a large portion of this residue was not hydrolyzed by treatment with 2 N HCl. Uronic acids made up a significant part of fractions S-1, S-2, and S-3 of *Staurastrum* sp., and these may be in the form of pectic substances since the only uronic acid found was galacturonic acid. A lignin-like material was found in fractions I-3 and S-4, the quantity of this substance in the two fractions being equivalent to 5.6 and 0.3%, respectively, of the wall weight. This lignin-like substance was not observed in the other fractions or in the

**Table 4. Composition of fractions obtained from walls of three algae**

| Alga            | Fraction | Carbohydrates in acid hydrolyzates* | Soluble carbohydrate* | Constituents in acid hydrolyzates* |
|-----------------|----------|-------------------------------------|------------------------|-----------------------------------|
|                 |          |                                     |                        | Reducing sugars* | Uronic acids* | Not hydrolyzed* |
| *Staurastrum*   | S-1      | Gl(++, Ga(+))                       | 2.0                    | 0.2 0.8 0 0  |
|                 | S-2      | Gl(+++), Ga(+)                      | 4.0                    | 1.3 1.2 0 0  |
|                 | S-3      | Gl(+++), Ga(+)                      | 4.0                    | 1.0 0.4 0 0  |
|                 | S-4      | None                                | 1.0                    | 0.0 0.0 0 0  |
|                 | I-3      | Gl(+++), M(+), X(+)                 | 0.0                    | 32 0.0 40 |
| *Fischerella muscicola* | S-1 | Ga(+)                              | 0.5                    | 0.0 0.1 0 0  |
|                 | S-2      | Gl(++), M(++, X(++, F(, Ga(+)      | 68                     | 55 2.5 0 0  |
| *Pediastram duplex* | S-1  | Ga(+)                              | 0.3                    | 0 0.4 0 0  |
|                 | S-2      | X(++, Ga(++)                        | 27                     | 6.7 1.0 0 0  |
|                 | S-3      | Ga(+), F(+)                         | 8.2                    | 1.1 0.3 0 0  |
|                 | S-4      | X(++, Ga(+)                         | 8.4                    | 0.9 0.4 0 0  |
|                 | I-3      | Gl(+++)                             | 0.0                    | 87 0.0 7.5 |

*Relative abundance (+, ++, ++++) of glucose (Gl), galacturonic acid (Ga), mannose (M), xylose (X), and fucose (F).

*As percentage of wall weight.

*Treated for 6 h with 2 N HCl at 105°C.*
other algae. Protein was not found when the *Staurastrum* sp. fractions were subjected to hydrolysis with 1 N NaOH for 1 h.

The composition of *F. muscicola* walls was quite different from that of the other algae. Most of the material subjected to fractionation appeared in either the HCl-soluble S-2 or the NaOH-insoluble I-3 fractions (Table 4). These two fractions were quite similar in the kinds and amounts of materials which they contained, although alkaline hydrolysis of the two fractions followed by the Folin protein determination showed that fractions S-2 and I-3 had 0.2% and 3.1%, respectively, of the wall weight in the form of protein. The major components in hydrolyzates of fractions S-2 and I-3 were reducing sugars. Alkaline hydrolysis followed by protein determination (13) showed that fractions S-1, S-3, and S-4 contained an amount of protein equal to 0.2, 0.1, and 0.2%, respectively, of the total wall weight.

Fractionation of the walls of *P. duplex* yielded results similar to those expected in the fractionation of walls of higher plants, for which the method was devised (6). Galacturonic acid was an important component of fractions S-1 and S-2, whereas the only reducing sugar in S-2 was xylose. Because the anthrone test that revealed the abundance of soluble materials in S-2 is primarily a test for carbohydrates, xylose was probably the principle monomer responsible for the 6.7% value. Fractions I-3 and S-4 contained sporopollenin in amounts equal to 2.4 and 1.2% of the wall weight, but none of the other fractions and neither of the other two algae contained sporopollenin. After hydrolysis, fraction S-3 was found to have a reducing sugar component (fucose) in nearly four times the abundance of the uronic acid (galacturonic acid). Fraction S-4 had the monomeric composition which would be expected of a polyuronide hemicellulose, whereas fraction I-3 consisted almost entirely of glucose. Part of fraction I-3 could not be hydrolyzed with acid, a substantial portion of this unhydrolyzed material probably being sporopollenin. Alkaline hydrolysis of the fractions followed by the Folin protein test showed that fractions S-1, S-2, S-3, S-4, and I-3 of *P. duplex* contained 0.1, 0.3, 0.2, 0.1, and 0% of the total wall weight as protein, respectively.

**X-ray diffraction analysis.** To provide further evidence for the occurrence of cellulose, X-ray diffraction analysis was performed on dried slide mounts of the I-3 fractions of the algae and of MN cellulose 300 (Brinkman Instruments, Westbury, N.Y.) using the method of Gunnison and Alexander (Can. J. Microbiol., in press). The X-ray patterns are depicted in Fig. 1. Authentic cellulose showed broad peaks with maxima at 15 and 22 degrees 2θ and a small deflection having its maximum at 34 degrees 2θ. I-3 fractions of *F. muscicola* and *P. duplex* gave none of these patterns, thus indicating that cellulose was either not present or was masked by other components in these preparations. The I-3 fraction of *Staurastrum* sp. exhibited a 22-degree peak in the same region as the cellulose standard, suggesting that this polymer was present.

**Resistant components of cell walls.** Sporopollenin was isolated by the same procedure from the spores of *Lycopodium clavatum* (obtained from J. Russell, Cornell University), the "classic source of sporopollenin" (8), and from the walls of *P. duplex*. The infrared spectra of these preparations and of the acetylated derivative of *P. duplex* cell walls prepared by the method of Atkinson et al. (2) are given in Fig. 2. The spectra of the sporopollenins obtained from the walls of *P. duplex* and from *L. clavatum* are nearly identical. Almost the same spectrum was obtained using acetylated *P. duplex* walls. Since sporopollenin is the only known polymer of biological origin which can withstand the acetylation process (8, 21) and because the infrared spectrum of *P. duplex* is the same as that of *L. clavatum*, the preparation derived from *P. duplex* is considered to be sporopollenin. Analysis of the walls obtained from a 21-day-old *P. duplex* culture showed they contained 3.3% sporopollenin.

Lignin-like material was obtained from *Staurastrum* sp. in an amount equal to 5.6% of the wall weight. The pyrolysis-gas chromatograms of lignin from *Staurastrum* sp. and pine
wood meal are shown in Fig. 3. The patterns are nearly identical, except that the Staurastrum sp. preparation yielded a smaller quantity of products per unit of material pyrolyzed and only the algal preparation showed a small peak at about 4 min after initiation of pyrolysis. Infrared spectra of the two lignins were nearly identical except for a small shift of pattern between 1,400 and 1,800 cm⁻¹ and slight differences in the peaks occurring in the region between 800 and 1,400 cm⁻¹ (Fig. 4).

The authentic and the algal lignins were subjected to alkaline fusion by a slight modification of the method of Rassow and Zickmann (20). To 2.5 g of KOH and 2.5 ml of distilled water in a nickel crucible were added 10 mg of the preparation and 90 mg of zinc dust. The mixture was heated on a steam bath either for 10 min or until the KOH had dissolved. The mixture was brought to 250 °C in a furnace, at which time 5.0 ml of a 50% KOH solution in distilled water and an additional 0.75 g of zinc dust were added. Heating at 250 °C was continued for 1.5 h, after which the mixture was cooled to room temperature and dissolved in 40 ml of distilled water. The alkali-fused lignin was acidified to pH 2.5 with 6 N HCl, and the mixture was dried on a rotary evaporator and then dissolved in 5.0 ml of distilled water. Residual KCl was removed by filtration. Using the procedures described above for analysis of the fused lignin, it was observed that protocatechuic acid and the products of alkaline fusion of the authentic and the algal lignins had Rf values of 0.39 by thin-layer chromatography and retention times of 39 s by gas chromatography. Thus, the Staurastrum sp. preparation apparently possessed a phenyl structure, a characteristic of lignins (20).

Decomposition of labeled walls and wall fractions. The degradation of Staurastrum sp. walls and wall fractions by soil microorganisms is presented in Fig. 5. Although the lignin preparation obtained from this alga was subject to microbial decomposition, it was attacked more slowly than the original walls and all of the other fractions. The lignin-like material accounted for less than 30% of S-4; its presence may explain the slower rate of attack on S-4 than the other fractions, but the degree of protection is obviously not great. I-3 was readily metabolized, possibly the result of its content of cellulose and the lack of influence of the small quantity (less than 4%) of lignin-like material present. Fractions S-1, S-2, and S-3 were rapidly degraded, and less than 25% of the original ¹⁴C label remained after 24 days. Since the quantity of ¹⁴C left in the fractions after 24 days was directly correlated with their content of apparent lignin, it is possible that the constituents metabolized in the first week are largely
polysaccharides, leaving a refractory lignin-rich substance behind.

The decomposition of *F. muscicola* walls and wall fractions is depicted in Fig. 6. It is evident that S-1, S-3, and S-4 were rapidly degraded, each losing more than 50% of the original 14C label in 24 days. The rates of decomposition of fractions S-2 and I-3 were quite similar, and they were much more resistant to decomposition than the other fractions. The unfractionated wall material was especially refractory, and more than 80% of the original label was still present after 24 days. It is noteworthy that the rates of decomposition of S-2, I-3, and unfractionated walls were quite similar after the first few days, suggesting a possible role for components of S-2 and I-3 in shielding the walls from microbial attack. The rate of metabolism of [14C]glucose was determined to illustrate the rate of oxidation of a good carbon source for microorganisms.

The decomposition of labeled walls and wall fractions of *P. duplex* is shown in Fig. 7. The sporopollenin component was not attacked at all in the 24-day interval since all of its 14C label was recovered at the end of the test period. Fraction S-4 was more extensively degraded, but 50% of its original label was still present after 24 days. Unfractionated walls were even more readily decomposed, with slightly less than 40% of the original material left at 24 days. The other fractions were quickly metabolized within the test period.

**Effect of age on resistance.** *Staurastrum* sp. and *P. duplex* were grown as usual for 18 to 23 days in 16 liters of medium, and the cells were harvested aseptically by allowing them to settle for 6 h at 4 °C and then collecting the algae by centrifuging the cells that settled out; for this purpose, sterile bottles were employed. The algae thus obtained were added to 16 liters of fresh medium. The incubation was continued, and the cells were collected after an additional 5, 12, or 21 days of incubation. The organisms were washed twice in 0.01 M phosphate buffer (pH 7.0) and collected by centrifugation, and the empty cell walls which had been shed by the growing algae were removed from the surface of the algal pellet with a stream of buffer. The cells were then resuspended in fresh buffer and centrifuged, and the remaining old walls were removed by the same process until no further wall material could be obtained. The cells were then suspended in 100 ml of buffer. One-half of the suspension was used to prepare algal lawns as described by Gunnison and Alexander.
nol. Oceanogr., in press), whereas the remainder was lyophilized and used to prepare cell walls. The lawns were examined for their susceptibility to microbial attack by the method of Gunnison and Alexander (Limnol. Oceanogr., in press), and the lignin content of *Staurastrum* sp. walls and the abundance of sporopollenin in *P. duplex* walls were determined.

The data demonstrate a relationship between the lignin content of *Staurastrum* sp. walls and the decomposability of the alga (Table 5). The correlation is not linear because the major increase in lignin content occurred between 5 and 12 days, during which time the alga remained moderately susceptible, whereas the lignin level increased only slightly between 12 and 21 days during which period the cell became markedly resistant. The results with the sewage inoculum, in which a decrease in resistance occurred between day 5 and day 12, are anomalous.

Studies of the decomposition of *P. duplex* cells of various ages strongly suggest a relationship between the sporopollenin content of the wall and the decomposability of the alga. Although the 5-day-old cells were highly to moderately susceptible to degradation, 12-day-old cells were quite resistant (Table 5). During the 3-day period, the sporopollenin content of the walls rose from 1.2 to 4.2%. No difference in resistance was observed between the 12- and 21-day-old cells, and the sporopollenin content during this period remained reasonably constant.

**DISCUSSION**

Cellulase and polygalacturonase preparations act on *Chlamydomonas reinhardii* and *Ulothrix fimbriata*, two algae that are readily susceptible to attack in natural waters, and the preparations extensively degrade the algal walls so that the cells are converted to spheroplasts (Gunnison and Alexander, Can. J. Microbiol., in press). By contrast, the cellulase and polygalacturonase preparations exhibited low activity against the resistant algae. These data thus correlate with the resistance of the organisms in freshwaters (Gunnison and Alexander, Limnol. Oceanogr., in press).

The observation that moderate digestion of *P. duplex* walls was affected by the two enzyme preparations, as contrasted with the small influence on intact cells, suggests that enzymatic hydrolysis of the surface of this organism is facilitated by the exposure of areas of the wall other than the outer surface. Inasmuch as such exposures probably occur in nature only when the cell is perforated mechanically or when autolysis occurs, the polysaccharidase activity probably is not often important in governing the persistence of the alga. Although the polygalacturonase preparation was not free of cellulase, the release of galacturonic acid from *P. duplex* walls which were incubated with this preparation suggests that the walls contain a polysaccharic acid, the latter possibly serving to hold together either fibrils or layers of cellulose. The failure of lysozyme to digest walls of these resistant species is in contrast with the susceptibility of *Cylindrospermum*, a lysozyme-sensitive blue-green alga quite susceptible to microbial decomposition in freshwaters (12; Gunnison and Alexander, Limnol. Oceanogr., in press).

The walls of *Staurastrum* sp. contain a component which appears to be similar if not identical with lignin, a polyaromatic known for its resistance. The slow decomposition of the purified microbial lignin or lignin-like material, as compared with the rate of degradation of the other fractions or the unfractionated walls, suggests that the polyaromatic may be responsible for the slow rate of decomposition of the alga in nature. The absence of a linear correlation

### Table 5. Effect of age on the susceptibility of two algae to microbial attack and on their lignin and sporopollenin content

| Species          | Age of alga (days) | Days for decomposition to be evident | % of wall weight as: |
|------------------|--------------------|-------------------------------------|----------------------|
|                  | Honeoye silt loam  | Field soil* | Garden soil* | Swamp sediment* | Sewage* | Lignin | Sporopollenin |
| *Staurastrum* sp.| 5                  | 6         | 7           | 7              | 10      | 10    | 1.2       |
|                  | 12                 | 12        | 12          | 8              | 13      | 6     | 4.6       |
|                  | 21                 | >30       | 28          | >30            | >30     | >30   | 5.6       |
| *Pediastrum* duplex | 5              | 4         | 5           | 13             | 13      | 12    | 1.2       |
|                  | 12                 | >30       | >30         | >30            | >30     | >30   | 4.2       |
|                  | 21                 | >30       | >30         | >30            | >30     | >30   | 3.9       |

*Described by Gunnison and Alexander (Can. J. Microbiol., in press).*
between content of apparent lignin and decomposability may signify that some other component determines resistance, although the last lignin deposited by the organism may serve as the shield against microbial attack.

The data strongly suggest but do not show unequivocally that the material isolated from *Staurastrum* sp. walls is in fact lignin. The limited amount of information on the possible occurrence of lignin in algae is usually interpreted to mean that it is not present (17), although some data exist to the contrary (9). On the other hand, lignin in a group as primitive in the evolutionary sequence as the algae might differ substantially from the polymer characteristic of higher plants.

*F. muscicola* was resistant not only in natural waters but its walls also were totally unaffected by the enzymes used. Nevertheless, three of the cell wall fractions were easily degraded, so that they probably do not contain the substance conferring resistance. Fractions S-2 and I-3 decomposed quite slowly and at approximately the same rate, and hence they may contain the refractory constituent. S-2 and I-3 had approximately the same monomer composition, and it thus seems reasonable to believe that resistance of the walls and of the alga are related to constituents of fractions S-2 and I-3. Although these fractions were metabolized to a slight degree before the rate of decomposition declined to almost that of the original walls, this initial attack may have been at the expense of inaccessible components made available by the fractionation procedure. Because acid hydrolyzates of these two fractions contained several different sugars in similar amounts as well as galacturonic acid, it is tempting to postulate that the resistance is attributable to the presence of a heteropolysaccharide.

Sporopollenin but none of the other fractions of the walls of *P. duplex* was completely refractory to microbial attack. Thus, sporopollenin seems to be responsible for the resistance of the walls and presumably the persistence of the alga. Evidence exists that the sporopollenin is localized in the outermost layers of the wall of *Pediastrum* (2, 16). Silica is also present on the surface of *Pediastrum* walls (14), and the silica may likewise protect other components of the wall and thus shield the cell contents from microbial attack. In view of the digestion of the walls and the release of glucose by the cellulase preparation, cellulose is apparently present in *P. duplex*. This polymer may be located in fraction I-3, which yielded large quantities of glucose on acid hydrolysis.

The results demonstrate that the biochemical bases for resistance of at least certain algae to microbial attack in natural habitats can be attributed to discrete structural entities. In addition, resistance of the wall to decomposition has an important effect on the rate of mineralization of its several constituents. Why polyaromatics and heteropolysaccharides may contribute to the longevity of the algae has already been discussed (1). Whether these constituents are important to the survival of other species and if different wall components account for the resistance of other algae to microbial decomposition require additional investigation.

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