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The Composition and Development of Cell Walls of *Fucus* Embryos

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Summary. Changes in the structure and composition of cell walls of *Fucus gardneri* Silva were related to embryo development. Results of histochemical treatments of walls from embryos of different ages were compared with those of differential extractions and electron-microscopic examinations of isolated walls. At 24 h after fertilization alginic acid, fucoidin and cellulose were structural constituents of the embryo wall. The distribution of alginic acid and cellulose was uniform over the cell walls of embryos of any age, but fucoidin was concentrated at the rhizoid end of cell walls isolated from germinated embryos. Evidence is presented for the presence of sulfated xylogalactofucoglucuronan and β,1–3 glucan in the embryo cell-wall. Electron micrographs of untreated and differentially extracted cell walls showed that alginic acid comprised the fibrillar material and fucoidin comprised the amorphous material of the wall.

Fucus eggs do not have cell walls and the zygotes began wall synthesis within 40 min after fertilization. During the first 2 h there was a rapid deposition of alginic acid. After 4 h the wall thickness increased linearly through 24 h. Embryos treated with cycloheximide ceased wall growth after 6 h. Wall isolated from 24-h-old cycloheximide-treated embryos resembled wall isolated from control embryos 2–4 h old.

Introduction

The shape of the cell wall largely determines the shape of plant cells. Localized changes in wall composition that affect the mechanical properties of the wall may be the primary events necessary for morphogenetic responses involving changes in both cell size and shape. Embryos of the marine alga *Fucus* are excellent materials for studies concerned with the relationship between alterations of the wall and early morphogenesis of the zygote because the eggs are shed free of the parent plant and are without a cell wall. Wall synthesis begins within 40 min following fertilization (Pollock, 1970). The establishment of polarity occurs at about 12 h, followed by initiation and growth of the rhizoid (Jaffe, 1968; Quatrano, 1972). Elongation of the rhizoid, referred to as germination, causes the spherical zygote to develop into a polar, pear-shaped cell with a definite axis of symmetry. Since large numbers of embryos can be grown synchronously, and preparations of isolated walls can easily be obtained from embryos of different ages (Moon and Forman, 1972), changes in cell-wall organization and composition can be correlated with the development of the embryo.

While detailed chemical studies have not been done on isolated walls of brown algae, the total polysaccharides of these plants have been described by many authors (reviewed in Percival and McDowell, 1967; Bourne *et al.*, 1969; Bidwell

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et al., 1972). These studies have been on whole fronds and the cell wall is defined as the material remaining after extraction of the frond with dilute acid and base. Extraction with dilute acid and base removes, respectively, fucoidin and alginic acid, compounds that have been demonstrated to be present in the wall of *Fucus* (McCully, 1965–1970).

This report deals with the composition and development of the *Fucus* embryo cell-wall. These studes have involved differential extraction, electron microscopy, histochemical techniques, and the use of cycloheximide to study the relationship between protein synthesis and wall development.

Materials and Methods

Treatment of Embryos and Preparation of Walls

Embryos of *Fucus gardneri* Silva were obtained and walls isolated from them as previously described (Moon and Forman, 1972). Embryos designated as cycloheximide treated were derived from gametes released and fertilized in sea water plus 5 μg/ml cycloheximide. The culture of the embryos, so obtained, was continued in the presence of the drug until the embryos were used. Wall preparations were made from these embryos in the usual way, except that the centrifugation between homogenizations was carried out in a Serval centrifuge at 5000 rpm for 15 min. Cell walls from embryos treated with cycloheximide are very light and difficult to bring down by gentle hand-centrifugation.

Cell-Wall Extractions

Fucoidin was extracted from the cell walls by treatment with 0.1 N H_2SO_4 at room temperature for 3 days (Percival and McDowell, 1967), alginic acid by treatment with 3% Na_2CO_3 at 50° also for 3 days (Percival and Ross, 1948). To obtain walls from which both alginic acid and fucoidin had been removed, the walls were first extracted in 0.1 N H_2SO_4 and then in 3% Na_2CO_3. Cellulose is not removed by these treatments (Percival and Ross, 1949). Cell walls were also incubated in 0.1% RNAse (activity of the enzyme confirmed by measuring the hyperchromicity developed when incubated with a standard RNA solution) in water overnight at room temperature.

Histochemistry

Suspensions of cell walls, either untreated or extracted by one of the above procedures, were spread on glass slides coated with geletin adhesive (Jensen, 1962), dried on a slide warmer, and stained by one of the following treatments.

(1) Periodic Acid-Schiff's Reagent (PAS) (Jensen, 1962). Walls were oxidized 20 min in periodic acid and stained 15 min in Schiff's reagent. Since the periodic acid reacts only with those polysaccharides having free adjacent hydroxyls, cellulose and alginic acid will give a positive reaction and fucoidin will not (McCully, 1965).

(2) Toluidine Blue 0 (TBO, Matheson Coleman and Bell, Cincinnati, Ohio, USA) at pH 6.9 (McCully, 1965). Walls were stained with 0.05% TBO in 0.1 M phosphate buffer, pH 6.8, for 15 min, washed thoroughly in running tap water, and rinsed in deionized water. Both alginic acid and fucoidin stain positively.

(3) Toluidine Blue 0 (TBO) at pH 0.5 (McCully, 1970). Walls were stained in 0.05% TBO in HCl, pH 0.5, rinsed in 1 N HCl, washed thoroughly in tap water, and rinsed in deionized water. At this pH the carboxyl groups of alginic acid are not ionized, and this stain is considered specific for fucoidin (McCully, 1970).

(4) Azure B, pH 4.0 (Jensen, 1962). This basic stain is used to test for the presence of RNA and DNA.

(5) Unna, pH 4.4 (Jensen, 1962). This stain was prepared by dissolving 0.5 g of methyl green in 100 ml of 0.1 M acetate buffer at pH 4.4. The solution was extracted with chloroform to remove residual methyl violet. Then 0.2 g of pyronine B was dissolved in the solution of

methyl green. Suspensions of cell walls were stained from 1 to 3 min and then placed in a differentiating solution of tertiary butyl alcohol and absolute ethyl alcohol (3:1, v/v) for about 3 min. This mixture of methyl green and pyronine is usually used to stain nucleic acids.

(6) IKI-H_2SO_4 (Jensen, 1962). This is a standard procedure used to test for the presence of cellulose.

Extraction-Weighing Experiments

To determine the relative amounts of alginic acid and fucoidin present, a wall preparation isolated from 27 h embryos and weighing roughly 0.1 g was extracted by the above procedures and the percentage of the wall removed (by weight) after each extraction was calculated. The wall preparation was dried 2 days in a desiccator at room temperature, weighed, homogenized, extracted in 0.1 N H_2SO_4 to remove fucoidin, washed with water, dried, and reweighed. The walls were further homogenized and extracted in 3% Na_2CO_3 to remove alginic acid, washed, dried, and weighed again. Before washing, a small aliquot of each preparation was checked for the removal of alginic acid and fucoidin, using the appropriate histochemical tests.

Electron Microscopy

Whole embryos were fixed for electron microscopy according to Pollock (1970) in 2% glutaraldehyde (Ladd ampule), 2% formaldehyde (from *p*-formaldehyde), 0.15 M sucrose, 0.05 M cacodylate buffer, pH 7.4–7.6, for 12 to 20 h. The subsequent procedures were all carried out at 4°. Following fixation the embryos were washed for 4 h in buffer with decreasing sucrose concentrations, and placed in 2% OsO_4 in acetate-veronal buffer over a 2 h period. They were then stained with 2% uranyl magnesium acetate from 4 to 12 h, washed with deionized water, and placed in a 5-ml beaker with a small amount of water in a desiccator over pure acetone and $CaCl_2$ under reduced pressure (Sitte, 1962). After 2 days of dehydration the beakers were removed and the embryos given one change of acetone. A mixture of half acetone, half Spurr Epon (Polysciences, Inc., Warrington, Pa., USA) was dripped into the beaker with rapid stirring until the Epon concentration was about 15%. The acetone was allowed to evaporate and fresh Epon added. The embryos were infiltrated with Epon from 12 to 24 h, put into plastic capsules, and polymerization carried out at 40° for 24 h. Sections were cut with an LKB ultramicrotome, stained with 1% uranyl acetate and lead citrate (Pease, 1964), observed in a Philips electron microscope model 300, and photographed on 70-mm film.

Treated and untreated cell walls were prepared for electron microscopy by first drying the isolated walls on a millipore filter (Millipore Filter Corp., Bedford, Mass., USA), then wetting the filter and walls with water, and peeling the walls from the filter in a continuous sheet. Fixation and subsequent treatment were identical to that of whole embryos, except that sucrose was omitted from the buffer washes.

Determination of Cell Wall Growth

The thickness of embryo cell walls was determined by measuring micrographs of median sections of whole embryos cultured in sea water either with or without 5 μg/ml cycloheximide. Measurements were made at 2 h intervals for 0 to 24 h after fertilization. The average value from measurements of 10 profiles was used for each age. The electron microscope was calibrated with a carbon grating replica (Ladd), 21,600 lines/cm.

Results

Histochemistry

Untreated isolated walls from 24-h embryos stained deeply and uniformly with TBO at pH 6.8 (Fig. 1 A) and PAS (Fig. 1 E). When these walls were stained with TBO at pH 0.5 (Fig. 1 B) the rhizoid end was much more darkly stained than the thallus end. Cell walls from embryos with double rhizoids (e.g., Fig. 1 G) were stained in both rhizoid regions. Cell walls treated with RNAse did not differ

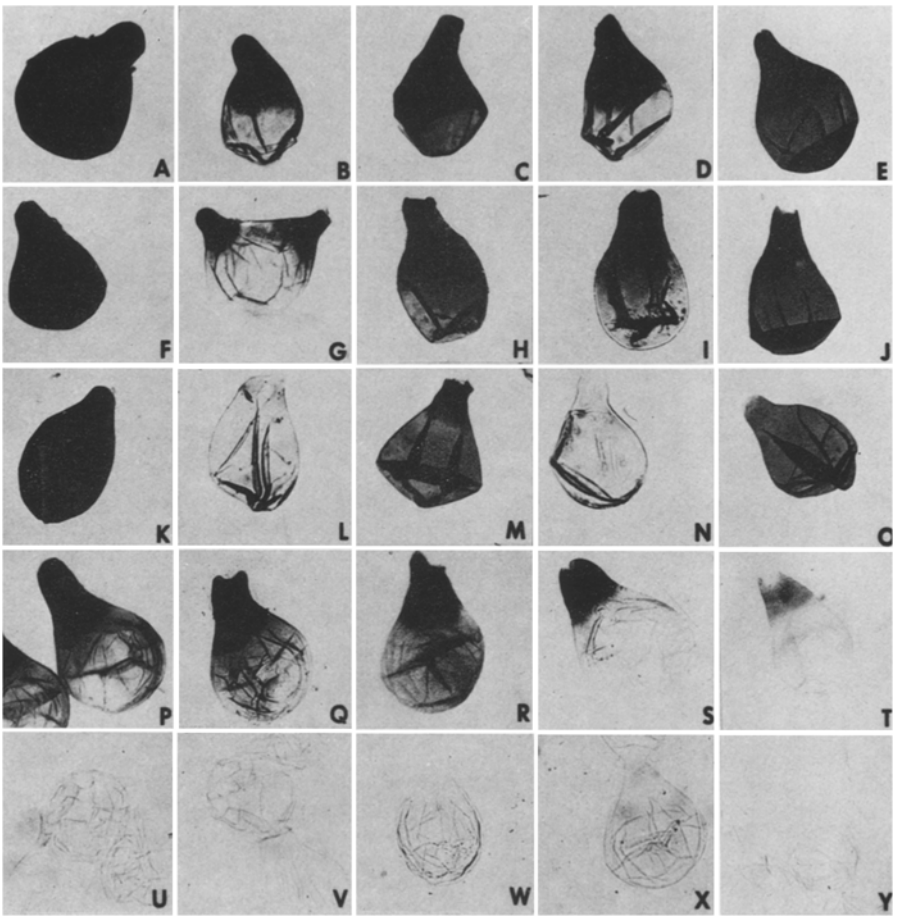

Fig. 1 A—Y. Summary of histochemical treatments of cell walls. × 160.
First column from left: walls stained with TBO at pH 6.8
2nd column: walls stained with TBO at pH 0.5
3rd column: walls stained with Azure B
4th column: walls stained with Unna
5th column: walls stained with PAS
Row A—E: walls untreated
Row F—J: walls treated with RNAse
Row K—O: walls extracted in 0.1 N H_2SO_4
Row P—T: walls extracted in 3% Na_2CO_3
Row U—Y: walls extracted in 0.1 N H_2SO_4 and 3% Na_2CO_3

from untreated cell walls in their reaction to any of the histochemical techniques used (Fig. 1 F—J, compared with A—E).

Cell walls extracted with 0.1 N H_2SO_4 to remove fucoidin exhibited a deep and uniform stain with both TBO at pH 6.8 (Fig. 1 K) and PAS (Fig. 1 O). The affinity for TBO at pH 0.5 (Fig. 1 L) was very slight and was not darker at the rhizoid end.

Cell walls extracted with 3% Na_2CO_3 to remove alginic acid exhibited similar polar staining patterns with TBO at both pH 6.8 (Fig. 1 P) and pH 0.5 (Fig. 1 Q).

There was little stain with PAS (Fig. 1T), but the color was slightly darker at the rhizoid end, indicating fucoidin may react slightly to this procedure.

Cell walls extracted with 0.1 N H_2SO_4 and then with 3% Na_2CO_3 to remove both fucoidin and alginic acid had little affinity for any of the above stains, but gave a positive reaction to the IKI-H_2SO_4 test for cellulose. A wet mount of these doubly extracted walls is difficult to see unless viewed with phase-contrast optics. In spite of the removal of these major polysaccharides, the walls did not disintegrate but retained the shape of the original embryo.

By using these specific histochemical techniques, patterns in wall changes were studied in relation to the overall development of the zygote. Cell walls of embryos less than 1 h old did not stain with TBO at pH 0.5, and there was little or no reaction to the IKI-H_2SO_4 test for cellulose. The walls stained positively with TBO at pH 6.8 and PAS, and disintegrated in 3% Na_2CO_3 but not in 0.1 N H_2SO_4, indicating the major, if not the only, component of these walls was alginic acid.

Embryos from 1 to 8 h old showed increasingly dark, uniform staining of the wall with both PAS and TBO at pH 6.8, indicating an increase in alginic acid. After 1 h, all cell walls gave a positive reaction to the IKI-H_2SO_4 test for cellulose and none disintegrated in 3% Na_2CO_3. The walls of embryos younger than 8 h showed little or no affinity for TBO at pH 0.5, indicating fucoidin is not a significant constituent of the cell walls of embryos of these ages.

After 8 h, increasing percentages of embryos had walls showing a localized deposition of fucoidin. By about 16 h nearly all the walls showed this fucoidin-rich area, and it was from this part of the cell that the rhizoid was always initiated. The walls from embryos 8 to 24 h old gave increasingly darker reactions to PAS. By 12 h the cell walls already stained very deeply with TBO at pH 6.8, and it was difficult to detect additional staining in the walls of older embryos.

It has been reported on the basis of histochemical criteria that there is an asymmetric distribution of RNA in *Fucus* embryos at the time of rhizoid initiation (Nakazawa, 1966; Nakazawa and Takamura, 1966). Because the distribution of RNA reported by these workers so closely resembles the distribution of fucoidin in *Fucus* cell walls, and because TBO is a basic stain that can be used to localize nucleic acids (Brachet, 1953), *Fucus* cell walls were treated with nucleic-acid stains to see if these basic dyes would also attach to acid polysaccharides.

Both Azure B and Unna stained fucoidin. Azure B also stained alginic acid. Unna at pH 4.4, interestingly, was specific for fucoidin and the stain was identical to the pattern observed with TBO at pH 0.5.

Cell-Wall Extractions

An initial extraction of cell walls from 24-h embryos with dilute acid removed 33% of the weight of the walls. Subsequent extraction removed 37% of the weight of the walls with dilute base. If the walls were initially extracted in dilute base 44% of the weight was removed, and an additional 25% of the weight was then acid extractible. The initial extraction, whether acid or base, removed 7–8% more of the wall than it would have, had it been the second treatment. This 7–8% is thus nonspecifically extracted, although it is not removed by distilled water. Divalent cations, such as calcium, have been proposed as cross linkages between alginic

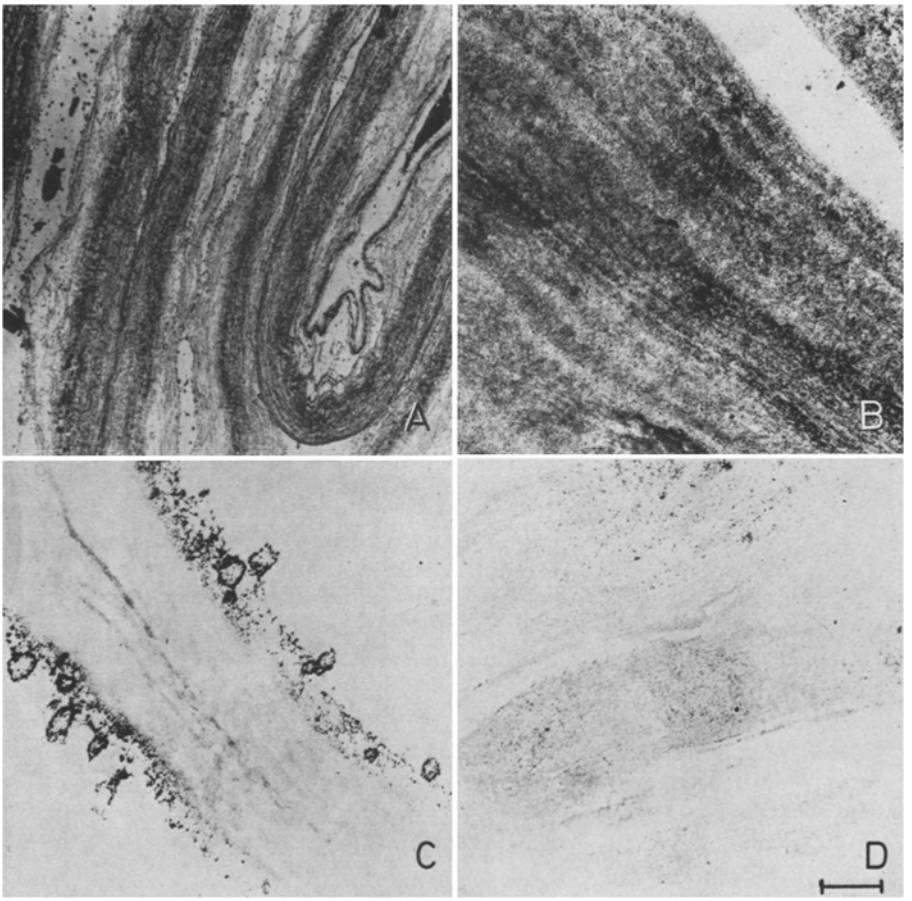

Fig. 2A—D. Isolated walls. (A) Untreated; (B) acid extracted; (C) base extracted; (D) acid and base extracted. Bar = 1 μm. × 8200

acid and fucoidin (Percival and McDowell, 1967) and such cations could be displaced by removal of either polysaccharide. The weight of the residue remaining after all extractions accounted for *ca.* 30% of the initial wall weight and was not affected by the order of the extractions.

Electron Microscopy

A comparison of electron micrographs of unextracted, isolated walls (Fig. 2A) with cell walls of fixed and sectioned embryos (Fig. 3B) showed that the cell wall is multilayered in both and the ultrastructure was not affected by the isolation procedure. Both these figures show cell walls that were composed of oriented, electron-dense fibrils. Cell walls that had been extracted in 0.1 N H_2SO_4 prior to treatment for electron microscopy (Fig. 2B) showed that the removal of fucoidin did not affect the electron density of the walls, but did disrupt the ordered array

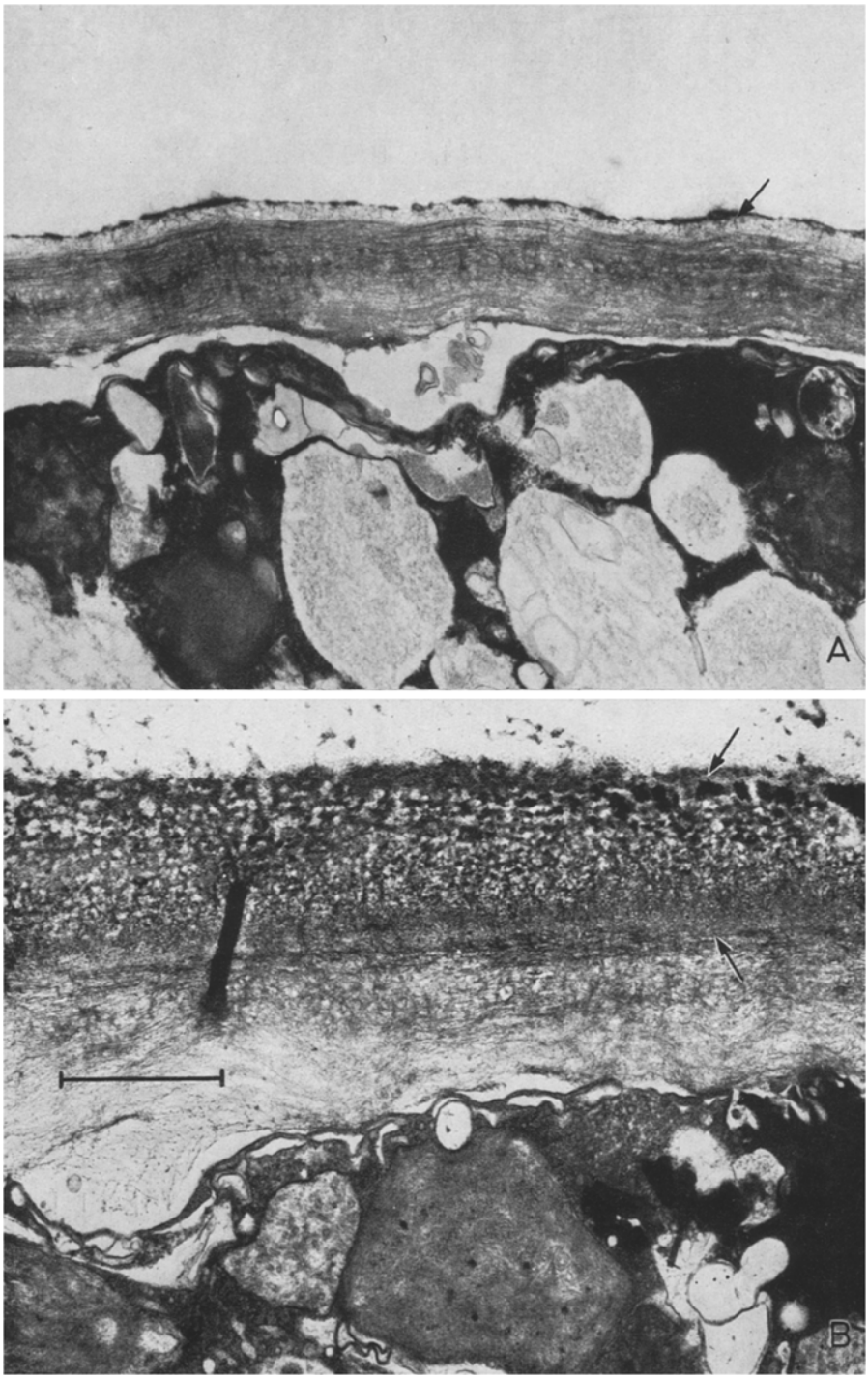

Fig. 3 A and B. Cell walls of whole embryos. (A) Wall at thallus; (B) wall at rhizoid. Arrows indicate amorphous material. Bar = 1 μm. × 22000

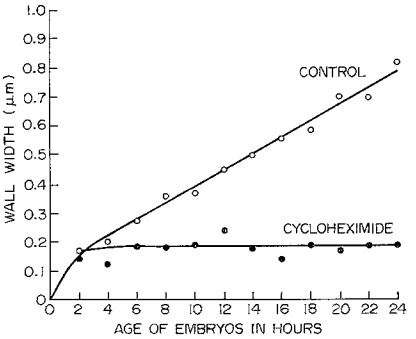

Fig. 4. Growth of walls from 0 to 24 h

of the fibrils. Cell walls that had been extracted in 3% $NaCO_3$ to remove alginic acid (Fig. 2C) showed greatly decreased electron density. The fibrillar material of the walls had largely been removed, and the only electron-dense material was the amorphous, darkly staining material at the outer surface of the wall. If the cell walls had been extracted with 0.1 N H_2SO_4 and then with 3% Na_2CO_3 (Fig. 2D), almost all of the electron density and fibrillar character of the walls was removed. Extraction with dilute base, whether or not it had been preceeded by extraction with dilute acid, removed the fibrillar component of the wall. The histochemical and ultrastructural evidence thus suggests that the fibrillar component is composed of alginic acid.

A comparison of the ultrastructure of the cell wall at the thallus end of the embryo (Fig. 3B) with the wall at the rhizoid end (Fig. 3A) showed that at 24 h the rhizoid portion of the wall was thicker and had additional amorphous material at the exterior. Since the amorphous material appears at 10–12 h, and it is extracted from isolated walls with dilute acid, we consider this component to be fucoidin.

Determination of Cell Wall Growth

Electron micrographs of *Fucus* embryos showed that from shortly after fertilization through 24 h the thickness of the cell walls was increasing. When cell walls were measured from micrographs, and the width of the fibrillar portion of the cell wall was compared to embryo age (Fig. 4) it was apparent that alginic acid was being continually incorporated into the wall. Wall material was rapidly deposited during the first 2 h, and then laid down at a linear rate through 24 h, reaching a thickness of 0.85 μm at that time.

Embryos that were released and cultured in cycloheximide also showed a rapid rate of wall deposition for the first 2 h following fertilizaiton; by 6 h, however, wall thickening had virtually ceased (Fig. 4). The cell wall attained a maximum thickness of 0.19 μm, approximately the same as in a 2–4 h embryo. A comparison of electron micrographs of 4-h embryos with those of 24-h cycloheximide-treated embryos (Fig. 5A and B) showed the ultrastructural similarity of these walls. Both are composed of oriented fibrils, and neither exhibited the

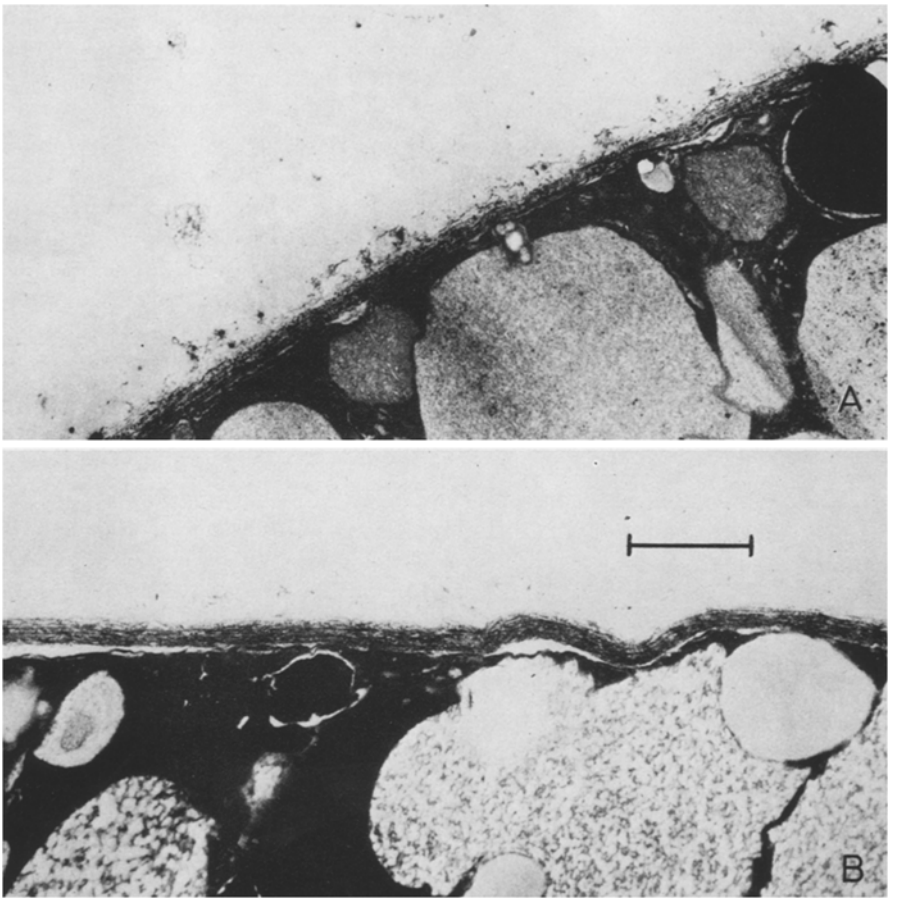

Fig. 5A and B. Cell walls of whole embryos. (A) 4-h control; (B) 24-h cycloheximide treated. Bar = 1 um. × 16000

darkly staining amorphous material. They were also histochemically indistinguishable, giving positive reactions for alginic acid and cellulose, and showing no indication of fucoidin. Cycloheximide itself did not interfere with fertilization; however, embryos cultured in cycloheximide did not germinate nor adhere to the Petri dishes as did the control embryos.

Discussion

The results of TBO staining at both acid and neutral pH indicated that alginic acid and fucoidin are major wall components. This agrees with previously published work dealing with chemically fixed cells walls of *Fucus* (McCully, 1970). Staining of unextracted walls of 24 h embryos with TBO at pH 0.5 was much darker at the rhizoid end than at the thallus end. But when these walls were stained with TBO at pH 6.8, the alginic acid stained uniformly over the wall and intensely enough to

mask the presence of fucoidin. Base extraction removes most of the PAS-staining material, indicating alginic acid is also responsible for most of the color developed by PAS. The PAS procedure is usually used to stain the total polysaccharides of the plant cell. In the wall of the *Fucus* embryo cellulose is present in such small quantities that PAS can be considered specific for alginic acid in this material.

Walls extracted with 3% Na_2CO_3 reacted very similarly with TBO at either pH 6.8 or 0.5, indicating that fucoidin is the only major charged polysaccharide remaining in these walls. Walls extracted with base gave a slightly positive PAS reaction, darker at the rhizoid. If fucoidin were incompletely sulfated, the occasional sugar residues lacking the sulfate would have a pair of adjacent hydroxyls open for attack by periodic acid. Also, even highly purified fucoidin contain traces of other sugars (Percival and McDowell, 1967) which might provide a small number of adjacent hydroxyls. However, the PAS stain in base-extracted walls was negligible, and fucoidin is essentially PAS negative.

Because fucoidin is a polyacidic molecule, it has special affinity for nucleic acid stains. Acetocarmine, used to stain nuclei, reacts with polysaccharide material at the rhizoid end of the embryo (Torrey and Galun, 1970). Our work demonstrated that both Azure B and Unna stained fucoidin. This staining may be the basis of the reports (Nakazawa, 1966; Nakazawa and Takamura, 1966) that RNA is concentrated at the rhizoid end of *Fucus* embryos. In these studies whole embryos were stained with Unna, and the resulting pattern resembles that of an isolated cell wall stained for fucoidin (Fig. 1B). In our experiments both Azure B and Unna were used at pH values at which alginic acid was ionized, but only Azure B stained alginic acid.

Other polysaccharides reported in *Fucus* include laminarin, which is water soluble and would not be found in isolated walls, and a polysaccharide containing xylose, galactose, sulfated fucose, and glucuronic acid (Bourne *et al.*, 1969; Bidwell *et al.*, 1972). The latter is extractable with dilute base but not with dilute acid, and consequently would be removed with alginic acid. Isolated walls that had been acid extracted retained a pale, persistent, uniform stain with TBO at pH 0.5. Walls extracted with dilute base lost this ability to stain, consistent with the idea that this heteropolysaccharide is a wall component.

The lack of TBO staining at any pH by cell walls extracted in both dilute acid and base indicated that all acid polysaccharides had been removed, including the above-mentioned sulfated xylogalactofucoglucuronan. Previous studies have demonstrated that small amounts of cellulose remain in fronds after extraction with dilute acid and base (Percival and Ross, 1949; Percival and McDowell, 1967). This cellulose could account for the faint PAS reaction and positive IKI-H_2SO_4 reaction. Our results, however, indicated that nearly one third of the wall weight remained after these extractions. If this residue were primarily cellulose a much stronger PAS reaction would be expected.

To remain unstained by any of the above histochemical procedures, any remaining polysaccharide could have neither acidic groups nor free adjacent hydroxyls. In whole fronds, the residue remaining after extraction with dilute acid and base is made up almost entirely of glucose units in β, 1–3 or β, 1–4 linkage (Bidwell *et al.*, 1972; Bourne *et al.*, 1969). A 1–3 linkage glucan would have no

adjacent hydroxyls, and would therefore be unstained by PAS. Thus it seems likely that this glucan forms a major fraction of the cell walls of *Fucus* embryos.

Differential extractions have previously been done on embedded and sectioned *Fucus* fronds (McCully, 1970; Parker and Diboll, 1966). There studies, however, have not involved embryos or materials prepared for electron microscopy. Alginic acid has previously been demonstrated in the fibrillar areas of the wall, but was interpreted to comprise the fine, rather discontinuous fibrils of the fringe of the filaments (McCully, 1965). Our results indicate that on the contrary, alginic acid comprises most of the fibrillar material of the wall of *Fucus* embryos.

The rate of wall synthesis is much greater during the first 2 h following fertilization than during the period of 4 to 24 h, and this initial wall is cycloheximide insensitive. Allen *et al.* (1972) have demonstrated that newly fertilized zygotes of *Pelvetia*, a close relative of *Fucus*, are in osmotic equilibrium with the environment until 3 h after fertilization. At this time the zygotes begin to take up inorganic ions, increasing their osmotic pressure until they germinate. The time course of these changes in *Pelvetia* corresponds to the measured wall changes in *Fucus*. Assuming the same osmotic phenomena to be occurring in *Fucus*, the internal pressure does not increase until the wall has developed sufficient strength to contain the developing forces. Likewise germination does not take place until a localized wall change allows the expansion of the rhizoid in response to the build up of internal osmotic forces.

The *Fucus* embryo offers some unique advantages for studying the control of wall formation. Two wall components, alginic acid and fucoidin, are easily differentiated by histochemistry and are also easily differentially extracted from isolated wall preparations. They are transported at different times to the wall. Alginic acid is rapidly laid down at the onset of wall formation and steadily thereafter over the whole cell surface. Fucoidin is deposited shortly before rhizoid initiation and in a highly localized area. Since these materials exist as high-molecular-weight compounds in cytoplasmic vesicles (McCully, 1968), the export of these vesicles must be controlled by two different mechanisms. It would be interesting to know if this control may be involved with the electrical current found in the zygote during rhizoid initiation (Jaffe, 1966–1970).

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