Plant Physiol. (1976) 58, 224-231

Cell Wall Assembly in *Fucus* Zygotes

I. CHARACTERIZATION OF THE POLYSACCHARIDE COMPONENTS[1, 2]

Received for publication February 18, 1976 and in revised form May 3, 1976

RALPH S. QUATRANO AND PATRICIA T. STEVENS[3]
Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331 and Marine Biological Laboratories, Woods Hole, Massachusetts 02543

ABSTRACT

Fertilization triggers the assembly of a cell wall around the egg cell of three brown algae, *Fucus vesiculosus, F. distichus,* and *F. inflatus*. New polysaccharide polymers are continually being added to the cell wall during the first 24 hours of synchronous embryo development. This wall assembly involves the extracellular deposition of fibrillar material by cytoplasmic vesicles fusing with the plasma membrane. One hour after fertilization a fragmented wall can be isolated free of cytoplasm and contains equal amounts of cellulose and alginic acid with no fucose-containing polymers (fucans) present. Birefringence of the wall caused by oriented cellulose microfibrils is not detected in all zygotes until 4 hours, at which time intact cell walls can be isolated that retain the shape of the zygote. These walls have a relatively low ratio of fucose to xylose and little sulfate when compared to walls from older embryos. When extracts of walls from 4-hour zygotes are subjected to cellulose acetate electrophoresis at pH 7, a single fucan (F_1) can be detected. By 12 hours, purified cell walls are composed of fucans containing a relatively high ratio of fucose to xylose and high levels of sulfate, and contain a second fucan (F_2) which is electrophoretically distinct from F_1. F_2 appears to be deposited in only a localized region of the wall, that which elongates to form the rhizoid cell. Throughout wall assembly, the polyuronide block co-polymer alginic acid did not significantly vary its mannuronic (M) to guluronic (G) acid ratio (0.33-0.55) or its block distribution (MG, 54%; GG, 30%; MM, 16%). From 6 to 24 hours of embryo development, the proportion of the major polysaccharide components found in purified walls is stable. Alginic acid is the major polymer and comprises about 60% of the total wall, while cellulose and the fucans each make-up about 20% of the remainder. During the extracellular assembly of this wall, the intracellular levels of the storage glucan laminaran decreases. A membrane-bound β-1,3-exoglucanase is found in young zygotes which degrades laminaran to glucose. It is postulated that hydrolysis of laminaran by this glucanase accounts, at least in part, for glucose availability for wall biosynthesis and the increase in respiration triggered by fertilization. The properties and function of alginic acid, the fucans, and cellulose are discussed in relation to changes in wall structure and function during development.

The developmental processes of growth, differentiation, and morphogenesis in plant cells are all intimately associated with alterations in cell wall metabolism. Because of the importance of these processes in understanding plant development, the structure of the cell wall has long been the object of intensive study (19). Relatively few studies, however, have been concerned with control of the initial assembly of a cell wall, or any one of the polysaccharide components, from a wall-less progenitor cell. Regeneration of cell walls around protoplasts, which have had the existing wall removed by a mixture of hydrolytic enzymes, has been investigated in yeast (15) and in several species of higher plants (2, 5). In the case of wall regeneration in cultured callus cells of sycamore, a polysaccharide component normally found in the wall is not assembled into the regenerated wall structure, but rather is secreted into the medium (5). Anomalous ultrastructure of regenerated walls has also been reported (9). In addition, regeneration has not yet been reported to be synchronous in large populations of cells, nor has the percentage of cells undergoing wall formation or the heterogeneous cell types involved been closely examined. Preliminary reports have shown the following characteristics of fertilized eggs of the brown alga *Fucus* to be ideal for a study of the control of cell wall formation around a wall-less egg cell in reponse to fertilization (12, 17, 25).

Fucus eggs have no detectable cell wall as evidenced by light microscopy (11). Within 30 to 45 min after fertilization a cell wall is present around every cell in a population of up to 10^8 zygotes. Time of fertilization can be controlled within ±15 min in dioecious species (*F. vesiculosus*) and ±30 min in monoecious species (*F. distichus, F. inflatus, F. seratus*). In such synchronously developing populations, the cell wall increases in thickness during the first 16 hr after fertilization. There is no cell growth of the zygote during this period, and cell division does not occur until 20 to 24 hr. Development of the zygote occurs free of other cells, aseptically, in a defined inorganic seawater medium with no light or external carbon source required, and under controlled laboratory conditions (21). In addition, methods for the isolation of purified cell walls have been reported (12, 14).

This paper presents a more detailed analysis of cell wall formation in *Fucus* zygotes and includes a method for isolating morphologically intact cell walls free of cytoplasm, measurement of the rate of new wall deposition, description of the polysaccharide composition of isolated walls at different stages of zygote development, and evidence for the possible role of a particulate β-1,3-glucanase in providing a source of energy and/or carbon for cell wall formation by hydrolysis of the storage glucan laminaran.

[1] This research was supported by Grant GB 37149 from the National Science Foundation and Grant GM 19247 from the Public Health Service and a fellowship from the Royal Norwegian Society of Science and Technology to R. S. Q. during a stay at the Institute of Marine Biochemistry, Trondheim, Norway.

[2] This paper and subsequent ones in this series are dedicated to the outstanding pioneering work in the field of algal polysaccharide chemistry by Professor Arne Haug (deceased May 1975) of the Institute of Marine Biochemistry in Trondheim, Norway.

[3] A portion of this research constitutes parts of a dissertation presented to The Graduate School of Oregon State University in partial fulfillment for the degree of Doctor of Philosophy.

MATERIALS AND METHODS

Receptacles of *Fucus distichus* (Newport, Ore.), *F. vesiculosus* (Woods Hole, Mass.; Trondheim, Norway), and *F. inflatus* (Trondheim, Norway) were treated according to previously published techniques for collection and incubation of zygotes in ASW[4] medium (21). Eggs of *F. vesiculosus* were labeled during oogenesis by incubating washed female receptacles in ASW containing $NaH^{14}CO_3$ (Amersham/Searle 59.9 mCi/mM). These receptacles were placed in a lighted incubator (1000 ft-c) for 48 hr at 15 C. The receptacles were removed from the incubator, washed several times in cold tap water to remove any gametes which may have shed during this period, and then stored 7 to 10 days at 4 C in the dark. At the end of this storage period, ^{14}C-labeled eggs were obtained from the stored receptacles according to standard procedures (21).

Cell Wall Purification. Embryos (1 cc packed cells) were homogenized in distilled H_2O in a power-driven Kontes Duall sintered glass homogenizer for 5 min and the volume of each sample brought to 10 ml. Cell walls and wall debris were collected from the homogenized sample by centrifugation (100*g*) with an International tabletop centrifuge. The pellet was washed three times with 10 ml of distilled H_2O. After resuspension in 10 ml of distilled H_2O, the washed pellet was sonicated for 30 sec every min for a total of 10 min in a Branson cell disruptor (model W 185) at a setting of 70 w. Walls were then collected by centrifugation, washed 5 to 10 times with distilled H_2O, and stored at −20 C until needed. About 10 mg dry weight of purified walls from 24-hr embryos were obtained from each cc of packed cells.

Chemical Analysis and Extractions. To determine the relative amounts of fucose containing polymers (fucans) and alginate in isolated cell walls, purified walls were sequentially extracted according to the procedure of Hogsett and Quatrano (8). Each extract was assayed using the cysteine reaction for fucose, the carbazole method for uronic acids, and the anthrone and phenol-sulfuric acid methods for total carbohydrate (8, 22). Over 93% of the total fucans and alginate in the initial wall preparations are extracted by this procedure and comprise about 75 to 80% of the total carbohydrate. All extracts obtained by this procedure were dialyzed, concentrated, and precipitated with alcohol for subsequent hydrolysis and sugar analysis, as well as for cellulose acetate electrophoresis.

The remaining alkali-insoluble material was treated with 10% (w/v) KOH at 110 C for 10 min. This extraction was repeated three times followed by five washes with distilled H_2O. The remaining pellet was referred to as the cellulose fraction. Solubility and staining properties, sugar analysis, and digestion with purified enzymes all indicate this fraction is cellulose (25). Although β-1,3 linkages were reported in this fraction (18), we have been unable to detect them chemically. The amount of carbohydrate in this cellulose fraction was determined by the anthrone and phenolsulfuric acid methods as well as by the semimicro method of Updegraff (28).

The relative percentage of the fucans, alginate, and cellulose in the total wall fraction was calculated from these values. At least 90% of the total carbohydrate in the initial wall preparation could be accounted for in these three polymer fractions using the above procedures and calculations. Protein content was calculated from nitrogen values determined on weighed samples of purified cell walls using a nitrogen analyzer.

To determine the amounts of mannuronic (M) and guluronic (G) acid, as well as the distribution of homopolymeric (MM and GG) and alternating (MG) blocks in the linear block copolymer alginate, wall fractions were hydrolyzed, neutralized, and chromatographed exactly according to previously published methods (6, 7, 24). Corrections were made for losses during hydrolysis (6). These conditions using strong acid were also sufficient for the hydrolysis of cellulose. Wall preparations were also hydrolyzed with weak acid, neutralized, and analyzed for neutral sugars by gas chromatography exactly according to previously described techniques (10). This weak hydrolysis does not appreciably hydrolyze cellulose or alginate, but does hydrolyze the fucans. Using these two different hydrolytic procedures on various fractions or whole cell walls, we were able to determine the sugar composition of the major polymers.

The procedure of Mian and Percival (13) was used to extract the storage glucan laminaran, which is not a cell wall component but located in the cytoplasm. The fraction solubilized by $CaCl_2$ was dialyzed overnight against deionized H_2O, and concentrated to a volume of 2 ml on a rotary evaporator. This fraction was applied to a cellulose (DE-52) microgranuluar, Whatman ion exchange column (1 × 15 cm) which had been equilibrated with 0.5 M KCl. The column was eluted with deionized H_2O (1.5 bed volumes). The eluant was dialyzed overnight against deionized H_2O, concentrated to 2 ml on a rotary evaporator, and made 80% with cold ethanol. This solution was stored at −20 C overnight to allow precipitation, then centrifuged at 10,000*g* for 10 min at 4 C. The white, ethanol-insoluble precipitate was resuspended in 2 ml of H_2O for determination of glucose and radioactivity.

Laminaran was hydrolyzed chemically by refluxing in 1 N HCl at 100 C for 3 hr, and enzymically using a purified endo-β-1,3-glucanase (supplied by E. T. Reese, United States Army Laboratories, Natick, Mass.). The reaction mixtures for enzymic hydrolysis contained a total of 0.5 ml which included 1 mg of purified enzyme in 10 mM acetate buffer, pH 4.8, and 5000 cpm ^{14}C-laminaran. Incubation was at 50 C for 24 hr. The reactions were terminated by centrifugation and storing the supernatants at −20 C. Aliquots of the reaction mixtures were spotted on Whatman 3MM filter paper discs, placed in 5 ml of Omnifluor (New England Nuclear), and counted in a Packard Tri-Carb liquid scintillation spectrometer (model 2425) at an efficiency of 88% for ^{14}C. Descending paper chromatography was used to resolve products of the enzyme hydrolysis of ^{14}C-laminaran. Of the mixture 200 μl were spotted on Whatman 3MM paper along with 10 μl of a 10 mg/ml solution of the following standards: glucose, cellobiose, and laminarabiose. Chromatograms were developed in 1-propanol-ethyl acetate H_2O (7:1:2) for 40 hr. The dried chromatograms were cut into strips (2 × 3 cm) and counted by liquid scintillation spectrometry. Standards were detected by silver nitrate (26).

Electrophoresis. To separate the different fucan polymers, about 1 to 3 μl of a 10 mg/ml solution of each sample was applied to a cellulose acetate (Oxoid) strip (5 × 9 cm) and subjected to electrophoresis at room temperature in 0.2 M calcium acetate (pH 7) for 3 hr at 2.5 mamp/strip (23). Strips were then stained for 3 to 5 min in 0.1% (w/v) TBO in HCl at pH 1 to 1.5 and followed by destaining in 7% acetic acid for about 10 to 15 min. Strips were then washed in tap H_2O, dried, and stored for recording.

Glucanase Preparation. Zygotes washed with ASW were homogenized in 0.1 M citrate phosphate buffer (pH 7) using 75 to 105 μm diameter glass beads (Sigma) in a Duall sintered glass homogenizer which was driven by an electric motor. The homogenate was filtered through two layers of Miracloth and centrifuged at 17,000*g* for 10 min. The pellet was resuspended in 0.1 M sodium acetate buffer (pH 4.8), to which was added 0.2 ml of a 1% aqueous Triton X stock solution to each ml of enzyme suspension. This suspension was incubated for 30 min and then centrifuged at 17,000*g* for 10 min. The supernatant was dialyzed against 2 liters of 10 mM sodium acetate buffer (pH 4.8) for 2 hr and used as a crude enzyme extract. All of the above procedures were done at 4 C.

[4] Abbreviations: ASW: artificial seawater; TBO: toluidine blue O.

Activity of the crude glucosidase was measured by its ability to hydrolyze various substrates. Laminaran (K and K Laboratories, Planview, N. Y.) and carboxymethylcellulose (Hercules Power Co., Wilmington, Del.) stocks were prepared at a concentration of 10 mg/ml in 0.1 M sodium acetate buffer (pH 4.8). ^{14}C-Labeled laminaran and ^{14}C-labeled cellulose, extracted from *Fucus* embryos according to procedures described above, and suspended in sodium acetate buffer (pH 4.8), were also used as substrates. When utilizing ^{14}C-cellulose and ^{14}C-laminaran as substrates, each reaction mixture contained 0.2 ml of labeled substrate (from 1000–4500 cpm) in addition to 0.3 ml of crude enzyme extract. Incubation was at 37 C for various times up to 24 hr.

Hydrolysis of unlabeled substrates was measured by the presence of reducing sugars as determined by the Park and Johnson method (27) using D-glucose as a standard. Hydrolysis of ^{14}C-cellulose was determined by filtering the reaction mixture immediately after incubation through a Whatman glass fiber (GF/A) filter and counting the filtrate in 10 ml of Aquasol (New England Nuclear) and the filter in 5 ml of Omnifluor. Hydrolysis of ^{14}C-laminaran was determined by subjecting reaction mixtures immediately after incubation to descending paper chromatography and radioactive determinations as described above.

Electron Microscopy. Fixation, dehydration, staining, and embedding eggs and zygotes was done exactly as described in a previous publication (20).

RESULTS

Properties of Cell Walls *in Vivo* and *in Vitro*. Cell walls were not evident in eggs of *F. vesiculosus* or young zygotes of *F. distichus* when plasmolyzed in 3 M NaCl, whereas 2-hr zygotes of both species always exhibited a cell wall under these conditions. Since fertilization can be more precisely timed in *F. vesiculosus*, a cell wall was observed in eggs as early as 10 to 15 min after addition of sperm. These results essentially confirm those of Levring (11) and were substantiated by electron microscopy. Vesicles from dictyosomes located in the peripheral cytoplasm of the egg became filled with fibrillar material after fertilization and emptied their contents into the newly formed cell wall (Fig. 1). A more detailed account of the origin, composition, and vesicle interrelationships during wall formation has been published (3).

Deposition of a cell wall was also detected by observing the appearance of wall birefringence (negative in the radial direction indicating ordered molecules arranged tangentially to the surface) when zygotes are viewed under plane-polarized light. Although a wall structure was detected by plasmolysis within 15 min in *F. vesiculosus*, wall birefringence was not observed until 60 min after fertilization, and not until 4 hr did the entire population of zygotes exhibit wall birefringence (Fig. 2). Treating zygotes with weak acid or base did not alter the birefringent

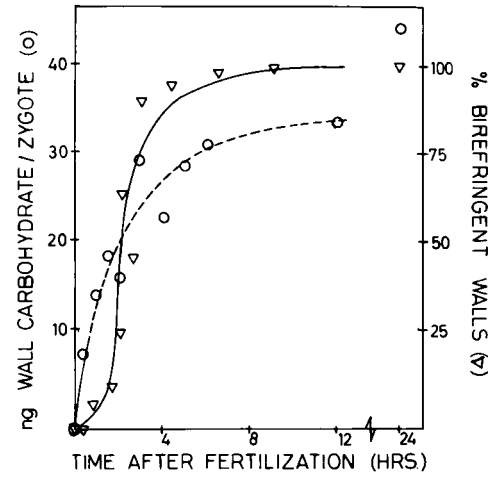

FIG. 2. Graph showing the time course of appearance of cell wall birefringence and the accumulation of carbohydrate in isolated cell walls of *F. vesiculosus* zygotes. Each point represents an average of at least 200 zygotes scored in each of three separate experiments for birefringence using a Zeiss polarizing microscope. Wall isolation and carbohydrate determinations were carried out as described in the text.

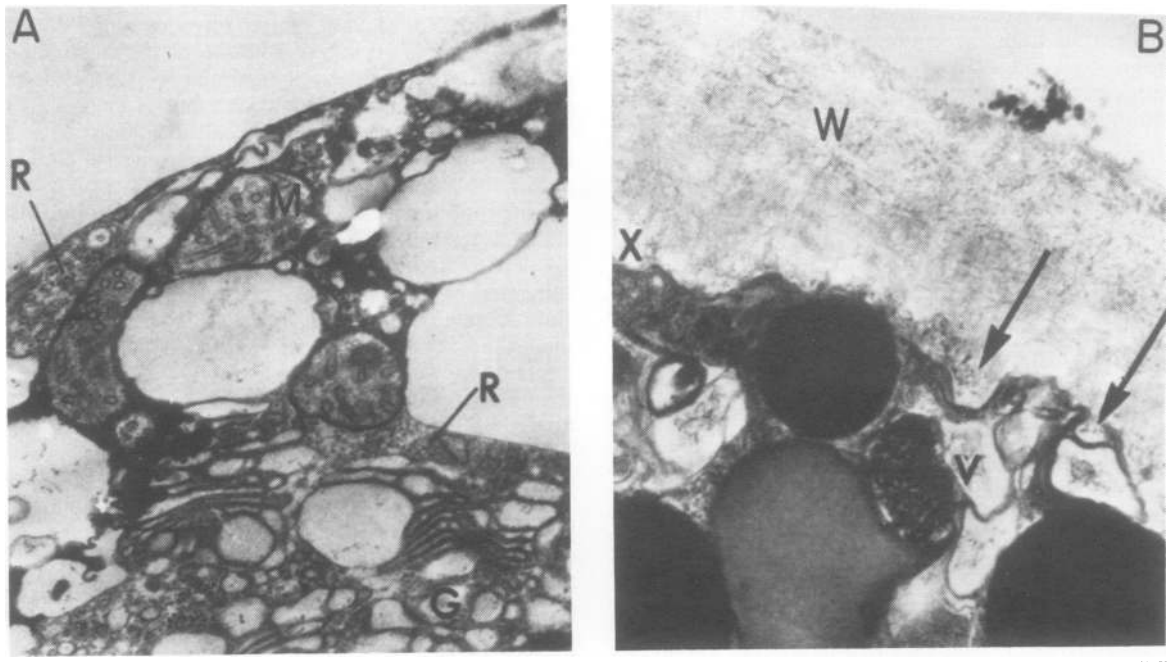

FIG. 1. A. Portion of an egg surface of *F. vesiculosus* stained with uranyl lead. No cell wall is evident but numerous mitochondria (M), ribosomes (R), and Golgi (G) are present just beneath the surface layer. 20,000×. B. Cortical region of the zygote of *F. vesiculosus*, 8 hr after fertilization, demonstrating the presence of a thick cell wall (W) when stained with uranyl lead. Arrows indicate what appear to be Golgi-derived vesicles emptying their fibrillar contents into the wall. Note also the close proximity of similar vesicles that have not yet released their contents (V), and remnants of ones having already deposited their fibrillar material (X). 40,000×.

properties of the wall, indicating that alginate (a polyuronide which exhibits birefringence and has been found in brown algal cell walls) is not directly responsible for the observed wall birefringence (1). Since cellulose has been isolated from embryos (25) and detected in the cell wall of *Fucus* embryos by specific cytochemical stains (11, 18), we believe cellulose is the wall polymer causing these birefringent properties, and hence deposited into an ordered structure between 1 and 4 hr after fertilization.

A method for obtaining intact cell walls from *F. vesiculosus* and *F. distichus* was developed that involved homogenization in distilled H_2O before and after brief sonication. Using this treatment, cell wall preparations were free of cytoplasmic contamination when observed under phase and dark field optics (Fig. 3). To quantitate the amount of cytoplasmic contamination in wall fractions prepared in this way, ^{3}H-fucose (2.22×10^7 cpm), a fucan-^{35}S (3.3×10^6 cpm), and a particulate fraction (30,000*g* pellet) from ^{14}C-labeled embryos (4.8×10^7 cpm) were added together and separately to the initial cellular homogenates from zygotes. After purification of the walls, at most 0.05% of the added radioactivity was still associated with the wall. Other methods of cell wall isolation that used more drastic chemical agents (12, 14) did not reduce this small amount of cytoplasmic contamination. No qualitative differences were observed in the polymer composition, birefringence, and staining properties of walls isolated in distilled H_2O when compared with *in vivo* walls (17, 18) or *in vitro* walls isolated by various other methods (12, 14). Purified cell walls obtained from embryos during the first 4 hr after fertilization, fragmented during the isolation procedure and did not retain the shape of the embryo. Cell walls isolated after 4 hr remained intact and over 75% of the population maintained the structural characteristics of a particular developmental stage (Fig. 4). This evidence coupled with *in vivo* birefringence, indicated a stable, ordered cell wall structure is assembled during the first 4 hr of embryogenesis.

Depostion of the cell wall *in vivo* could be followed by measuring the total carbohydrate and specific polymers in isolated walls at different stages of development. By 30 min, cell walls could be isolated in mg quantities and at 7 hr the total carbohydrate present was approximately equal to the amount in walls isolated at 14 hr (Fig. 2). By sequentially extracting cell walls of *F. vesiculosus* with weak acid, weak base, and strong alkali, the percentage of well characterized polysaccharide fractions could be determined (8, 13). The wall at 30 min possessed equal amounts of cellulose and alginate but fucans were not detected. By 4 hr, the composition of the wall was about 60% alginate, 20% cellulose, and 20% fucans (Fig. 5). This proportion of the major polysaccharide in the wall remained unchanged during the next 20 hr of development (Fig. 5) even though more polysaccharides were added during the period of rhizoid elongation between 16 and 24 hr (see Fig. 2).

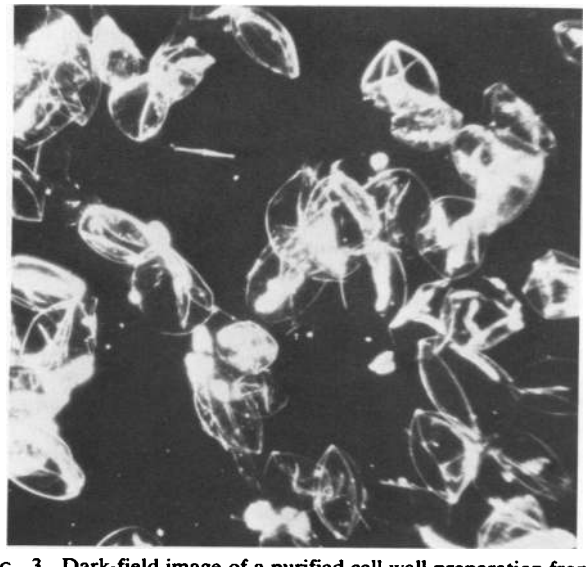

FIG. 3. Dark-field image of a purified cell wall preparation from 10-hr zygotes of *F. vesiculosus*. 100×.

After isolated walls were extracted so that only cellulose remained, the shape characteristics and birefringent properties were still evident. Cell walls isolated from 4- to 24-hr embryos remained intact even after treatment with 10% KOH at 100 C for 60 min (Fig. 4). Although cellulose was present as early as 30 min, a wall structure with characteristic shape and birefringent properties was not detected until 4 hr when all three major polysaccharide fractions reached stable proportions. After 4 hr, the shape and birefringence of isolated walls reside with cellulose, since the other polymers could be removed without the loss of these properties.

Cell Wall Composition. The protein content of 16-hr *F. distichus* walls was less than 3% by weight when determined by nitrogen analysis. Lipids and hexosamines were not detected in isolated walls of any of the species examined.

The neutral sugars and uronic acids found in 16- to 24-hr walls of embryos from *F. vesiculosus*, *F. distichus*, and *F. inflatus* were characteristic of those polymers found in brown algal cell walls, *i.e.* cellulose, alginate, and fucans. Weak acid hydrolysis, which does not appreciably degrade cellulose or alginate, yielded fucose-xylose-glucose-mannose-galactose in the ratio of 8:4:2:2:1. These ratios remained the same under strong acid hydrolysis with the exception of glucose which increased due to the hydrolysis of cellulose. Under these strong acid conditions, mannuronic (M) and guluronic (G) acids (components of alginate) were the major uronic acids found in cell walls. The M/G ratio varied between 0.33 and 0.55 indicating that the composition of alginate from purified cell walls has about twice as much guluronic as mannuronic acid. Partial acid hydrolysis of alginate isolated from 24-hr walls of *F. inflatus* was done to determine the relative proportions of the three types of uronic acid blocks in the polymer. The M-G heteropolymer blocks comprised 54%, while the homopolymer blocks of G-G and M-M yielded 30% and 16%, respectively. Glucuronic acid, a constitutent of fucans (10, 13), was found in cell wall hydrolysates when analyzed by column chromatography and later confirmed by paper electrophoresis and paper chromatography of the lactone derivative (6, 10).

Although no variation in uronic acid composition was observed in isolated cell walls from 3-, 16-, and 24-hr embryos of *F. vesiculosus* and *F. inflatus*, significant qualitative changes were found in the neutral sugar composition. The only sugar found in strong and weak acid hydrolysates of 2-hr walls was glucose. By 6 hr, the sugar components of the fucans were detected with fucose comprising the major sugar by the time of rhizoid formation at 14 hr (Table I). Fucose is initially incorporated into the cell wall between 2 and 6 hr and comprises an even greater proportion of the sugars present in walls at the time of rhizoid formation. Extraction of the fucans from the cell wall of 16 to 24 hr embryos of all three species yielded two major charged components when separated by cellulose acetate electrophoresis at pH 7. Two polymers with the same electrophoretic mobility were also found in the cytoplasm of these embryos. Neither component was detected in electrophoretograms from walls of 1-hr embryos. However, only the slower moving component (F_1) was initially detected in walls and cytoplasm from 1- to 6-hr embryos. A trace of the fast moving component (F_2) was first seen in the 6- to 10-hr cytoplasm but not in the cell wall until 12 to 14 hr. The appearance of F_2 in the wall at this time coincides with a shift in sugar ratios toward higher levels of fucose (Table I).

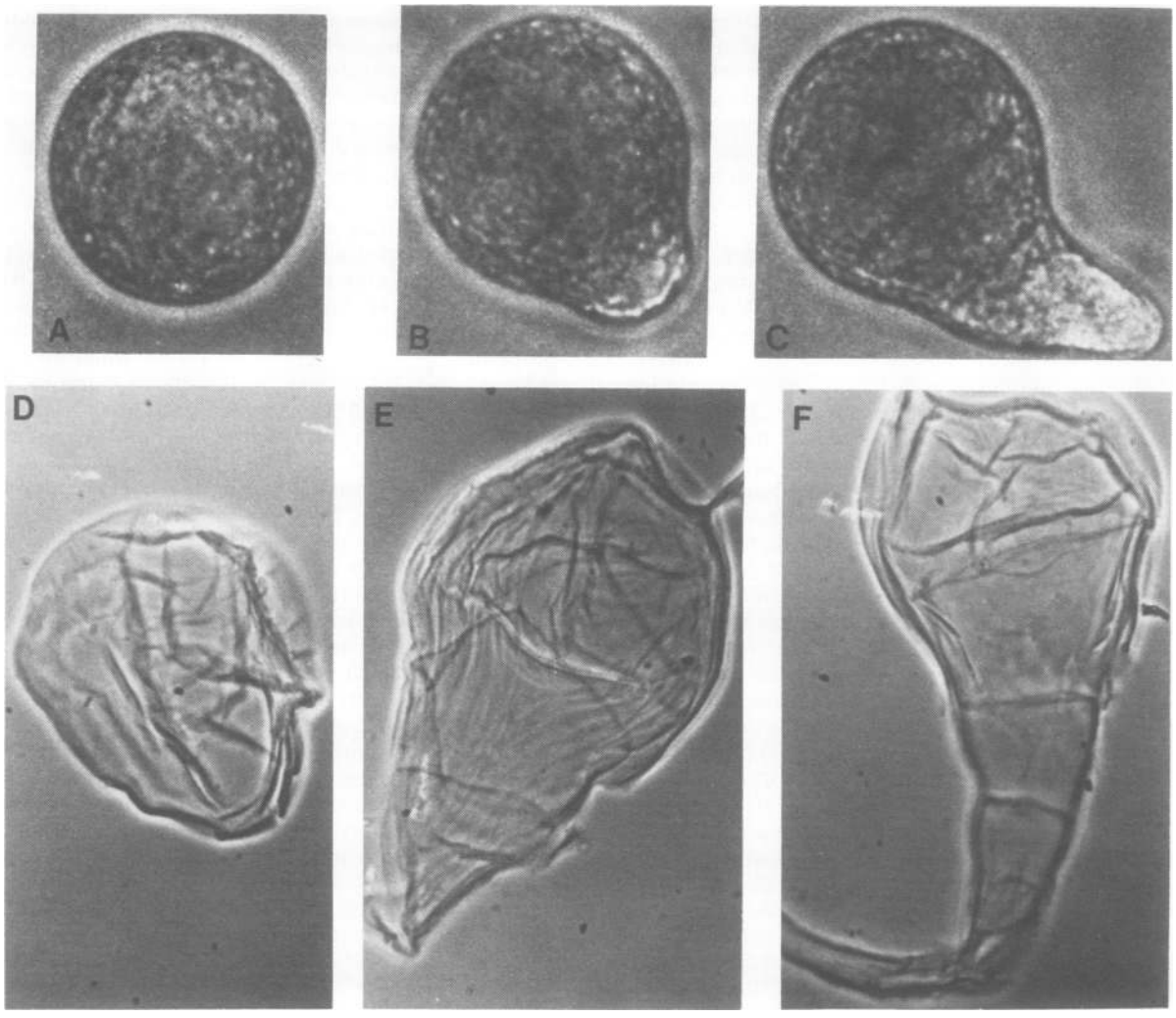

FIG. 4. Stages in the development of *Fucus* embryos: A: 0 to 12 hr; B: 12 to 16 hr; C: 30 to 36 hr. 1000×. (Compliments of Dr. G. B. Bouck.) Cell wall "ghosts" isolated from 4-hr (D), 24-hr (E), and 36-hr (F) embryos. These characteristic shapes are retained even after hot alkali extraction. 2000×.

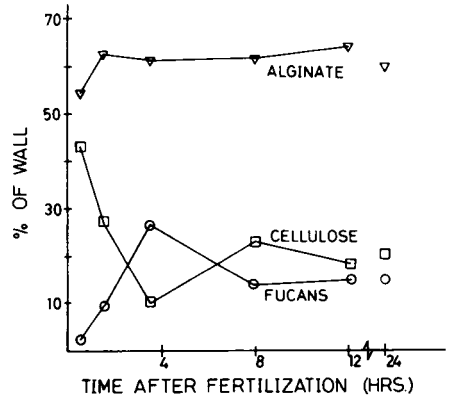

FIG. 5. Changes in the major polysaccharide composition of isolated cell walls from *F. vesiculosus* at different times after fertilization.

Substrates for Cell Wall Biogenesis. Polysaccharide deposition into the cell wall occurs in a defined inorganic medium containing few salts. Neither a carbon source nor light is required for wall development (21, 25). However, an endogenous energy source is required since *p*-trifluoromethoxyphenylhydrazone, an uncoupler of oxidative phosphorylation, prevents wall deposition and cellulose synthesis (25). Also, at least one of the cell wall polymers (cellulose) is newly synthesized as demonstrated by the incorporation of exogenously supplied $NaH^{14}CO_3$ or ^3H-glucose into an alkali-insoluble polymer (25). The carbon units and energy required for cellulose synthesis and wall formation may be supplied by dark CO_2 fixation, which has been reported in brown algae (16), or by hydrolysis of the storage polysaccharide in brown algae, laminaran.

When an aqueous $CaCl_2$ extract was prepared from *Fucus* embryos, applied to a DE-52 cellulose column, and eluted with distilled H_2O (13), an uncharged polysaccharide was obtained with the properties of laminaran. When this fraction was acid hydrolyzed, only glucose was detected upon chromatography. Treatment of this fraction with a purified endo-β-1,3-glucanase yielded only laminarabiose, with a small trace of glucose.

If the products of laminaran breakdown are used as substrates for the rapid cell wall synthesis following fertilization, a decrease in total cellular laminaran should occur during this period. Eggs of *F. vesiculosus*, previously labeled with ^{14}C during oogenesis, were fertilized and allowed to incubate in the dark for 7 hr in unlabeled ASW. The laminaran and cellulose fractions of these zygotes were extracted and compared to that from an equal volume of prelabeled, unfertilized eggs. As shown in Table II, both the total amount of laminaran and the radioactivity in this fraction decreased during the early period following fertilization, while the total amount of cellulose as well as the ^{14}C in cellulose increased. Since laminaran breakdown appears to occur con-

Table I. *Neutral Sugar Composition of Isolated Cell Walls*

Walls were hydrolyzed and analyzed by gas chromatography according to methods described in text. Scans from gas chromatograms were obtained and percentage of each sugar determined by calculating the area under the curves. Since the weak acid conditions employed do not appreciably hydrolyze cellulose or alginate, these figures approximate the sugar composition of the fucans. However, the glucose values may be abnormally high due to some hydrolysis of laminaran from cytoplasmic contamination.

SPECIES	TIME AFTER FERTILIZATION (hours)	FUCOSE	XYLOSE	GLUCOSE	MANNOSE	GALACTOSE
		% of total sugars				
F. inflatus	2	trace	trace	trace	trace	trace
F. vesiculosus	6	22	21	40	17	trace
F. inflatus	8	27	19	38	16	trace
F. distichus	16	45	27	10	16	2
F. vesiculosus	24	41	22	17	14	6
F. inflatus	24	46	24	13	12	5

Table II. *Changes in Amounts of Laminaran and Cellulose following Fertilization*

Eggs of *F. vesiculosus* were labeled with $NaH^{14}CO_3$ (2 μCi/ml) for 48 hr during oogenesis and then collected according to methods described in the text. One-half of the population was fertilized and incubated in unlabeled ASW for 7 hr, at which time laminaran and cellulose were extracted and analyzed. The other half of the egg population was treated in the same way except that they were not fertilized.

SAMPLE	RADIOACTIVITY IN LAMINARAN	AMOUNT OF LAMINARAN	RADIOACTIVITY IN CELLULOSE	AMOUNT OF CELLULOSE
	cpm	mg glucose	cpm	mg glucose
Unfertilized	100,080	42.5	6,560	0.13
Fertilized	40,810	11.9	28,840	0.73

comitantly with cell wall formation, carbon units produced could be used directly for wall biosynthesis as well as for ATP production.

Homogenates of *Fucus* embryos possessed β-glucosidase activity as measured by release of *p*-nitrophenyl from the synthetic substrate *p*-nitrophenyl-β-D-glucopyranoside (21). Approximately 80 to 90% of the β-glucosidase activity was found in a particulate fraction (40,000*g* pellet) and was released when treated with Triton X. When this solubilized enzyme preparation from *F. distichus* embryos was incubated with laminaran, reducing groups were released. No activity was found when this enzyme preparation was incubated with carboxymethylcellulose or ^{14}C-cellulose extracted from *Fucus* zygotes. To obtain a better characterization of the products released by this β-1,3-glucanase, ^{14}C-laminaran from *F. vesiculosus* was used as substrate. The soluble enzyme preparation from *F. distichus* zygotes released increasing quantities of glucose from ^{14}C-laminaran over a 24-hr period (Table III). Glucose was the only hydrolysis product detected in reaction mixtures. These results provide evidence that the enzyme is probably an exo-β-1,3-glucanase.

Table III. *Enzymatic Release of Glucose from ^{14}C-Laminaran*

A particulate enzyme was prepared from *F. distichus* embryos and assayed according to methods described in the text. All of the radioactivity released cochromatographed with a glucose standard, and at 24 hr 17% of the total cpm in the reaction mixture had been released. No radioactivity was associated with laminarabiose, which was clearly separated from glucose in the chromatographic system.

Time of Incubation (hours)	Glucose Released (cpm)
0	43
4	204
16	676
24	792
24 (-enzyme)	0

DISCUSSION

The cell wall from 24-hr embryos of *F. vesiculosus* consists of 60% alginate, 20% fucans, and 20% hot alkali-insoluble material (cellulose). Novotny and Forman (18) found about 40%, 30%, and 30%, respectively, in cell walls from 24-hr embryos of *F. gardneri* (= *F. distichus*). Differences between the two studies could be due either to the species used or to the methods employed. We determined the amount of fucose-containing polymers (fucans) and uronic acid-containing polymers (alginate) in both acid and basic extracts by specific colorimetric assays, which were then expressed as percentage of the total carbohydrate. The percentages reported for *F. distichus* represent the percentage of the dry weight in the acid (fucan) and basic (alginate) extracts (18). In either case, however, about one-half of the wall is composed of alginate and the remainder equally divided between the fucan and cellulose fractions. It also appears from this study that this cell wall is composed of a relatively small number of polymers; at least two electrophoretically distinct fucans, alginate, and cellulose.

Within the first 60 min after fertilization, a fragmented cell wall can be isolated which contains only alginate and cellulose. At no time did we observe a wall without alginate and cellulose. Fucose is not detected in hydrolysates of these early walls and when extracts are electrophoresed, fucans are not detected. However, between 1 and 6 hr after fertilization, fucose is detected in wall hydrolysates and when extracts of these walls are electrophoresed, only one fucan (F_1) is detected. The sugar composition of weak acid hydrolysates of the cell wall at this time indicates that F_1 resembles an "ascophyllan-like" (10) polymer, *i.e.* relatively high levels of xylose relative to fucose and low levels of sulfate. Preliminary results suggest that purified F_1 from embryos possesses these sugar ratios. Mian and Percival (13) describe a fraction with a similar composition to F_1 in an acid and an ammonium oxalate-oxalic acid extract from three other genera of brown algae. Because of its low concentration in walls and its low sulfate content, TBO staining procedures apparently cannot detect this fucan *in vivo*, since Novotny and Forman did not detect fucans in isolated walls of 6-hr *F. distichus* embryos (18).

When F_1 is incorporated into the wall during this stage of wall assembly, walls acquire birefringent properties and become sufficiently stable so that intact cell walls can be isolated and an internal pressure potential can be detected (21). Cellulose is

responsible for the birefringent properties of the wall and the delay in the appearance of birefringence (1–4 hr) in relation to the presence of cellulose in isolated walls (15–30 min) could be due to the low density of cellulose microfibrils in early wall assembly. However, the possible interaction of F_1 and cellulose in microfibril orientation and the acquisition of birefringent properties should not be overlooked. Although stabilization of the cell wall during isolation is coincident with the appearance of the fucan F_1, subsequent extraction of most of this polymer and all of the alginate does not result in collapse of the characteristic shape of the embryo wall (Fig. 4). However, even in the most highly purified samples of the cellulose fraction from embryo walls, we always find 5 to 15% of the neutral sugars to be comprised of equal quantities of xylose and fucose, the remaining being only glucose (25). Traces of xylose have also been reported in cellulose preparations from the brown alga *Himanthalia* (13). The fact that F_1 appears as the earliest matrix component in the developing wall, coincident with the acquisition of structural integrity and birefringence of the wall, tightly associated with cellulose, and to require harsh chemical treatment for removal, argues for its basic structural role in wall assembly and function.

By 12 hr the developing wall acquires the F_2 fucan which appears heavily sulfated due to its rate of migration upon electrophoresis and its characteristic metachromatic color upon staining with TBO. The fact that this new electrophoretic component is incorporated into the wall coincident with a change in sugar composition of the wall toward higher fucose levels argues against F_1, at a higher level of sulfation, accounting for the new electrophoretic species of fucan. We have also found that purified F_2 has two to three times more fucose than purified F_1, indicating that these two fucans are different sugar polymers (unpublished observation). This data along with previous work (8, 20, 21, 22) is consistent with the interpretation that F_2 is the fucan whose sugar chain is preformed in the cytoplasm of the egg (or early zygote) and is enzymically sulfated beginning at about 10 hr after fertilization. The sulfation occurs in vesicles derived from dictyosomes and is followed by the migration of the highly sulfated F_2 toward the rhizoid pole and its eventual incorporation into the elongating rhizoid wall. Since this study demonstrated that the percentage of the total cell wall composed of fucans at 24 hr is the same as before rhizoid elongation, it is possible that the fucan complement in the rhizoid wall consists of F_2 in place of F_1. In a recent microscopic study, Vreugdenhil *et al.* (29) showed that the elongating rhizoid wall has a much thinner outer fibrillar layer than the thallus wall, but possessed a unique fibrillar layer which stains metachromatically with TBO. Although it is clear from this indirect evidence that a sulfated F_2 is localized in only the rhizoid region, it is not known if an unsulfated F_2 (which would not stain metachromatically with TBO) is deposited at other sites in the embryo wall. It would be important, then, to determine if sulfation of F_2 is obligatory for the secretion and localization of this fucan at the rhizoid site. One approach to this problem would be to grow embryos in the absence of sulfate. Under these conditions, zygotes form "normal" rhizoids, but do not sulfate F_2 or adhere to the substratum (4). Lack of adhesion could be due either to the lack of the fucan in the rhizoid wall or the absence of a sulfated F_2 in the rhizoid wall. Unfortunately, no procedure now exists for the separation of the rhizoid and thallus cells, or for cytochemically distinguishing F_1 from an unsulfated F_2. Such procedures could directly demonstrate if the sugar complement of the fucans in the thallus and rhizoid cell wall are different, as well as to elucidate the importance of sulfation for the localization of F_2 and the presence of F_2 in the wall for adhesion of the embryo to the substratum.

The major constituent of cell walls from zygotes and embryos is alginate, a binary, linear heteropolymer containing 1,4-linked β-D-mannuronic (M) and α-L-guluronic (G) acid residues in varying proportions. These monomers are arranged in a blockwise fashion within the same chain as $(MM)_n$, $(MG)_n$, and $(GG)_n$ stretches (7, 24). It has been shown that the physical properties of alginates depend not only upon the monomer composition but also upon the relative proportion of these blocks (24). The solubility of alginate in acid, its ability to bind water, and the formation of flexible gels is particularly dependent upon the proportion of MG blocks in the polymer. The formation of clear, rigid gels with high mechanical strength depends mainly on the selective binding of Ca^{2+} to GG blocks forming autocooperative junctions (24). Alginate isolated from cell walls at different times after fertilization showed no significant variation in M/G ratio or in the percentages of the three types of blocks. The very low M/G ratio and block distribution consisting of about 85% MG and GG blocks indicates that the alginate is present as a transparent, flexible gel with high mechanical strength. It would be interesting to determine if wall assembly is initiated by the self-assembly of alginate into a gel on the surface of the egg triggered upon fertilization by the release of alginate from cytoplasmic vesicles (3) into the calcium-containing seawater. Also, does the stored alginate in the egg cytoplasm have the same M/G ratio as the alginate in the wall? Haug *et al.* (7) suggested that the biosynthetic route of alginate involves the formation of a mannuronan which is transformed at the polymer level by a C-5 epimerase into a heteropolysaccharide containing both types of uronic acids. Alginate from walls possessing a higher proportion of G relative to alginate from the cytoplasm may implicate a role for this epimerase.

It has also been shown in this study that carbohydrate deposition into the cell wall of *Fucus* zygotes occurs at a rapid rate in response to fertilization. These events take place in an inorganic medium with no external carbon or light source needed. An endogenous energy source is required since an uncoupler of oxidative phosphorylation (*p*-trifluoromethoxyphenylhydrazone) blocks wall assembly and the synthesis of at least one wall polymer, cellulose (25). The carbon units and energy required for this rapid wall formation may be derived from the enzymic hydrolysis of the storage glucan laminaran. Laminaran, isolated from the cytoplasm of *Fucus* zygotes, decreased during the first 7 hr of wall assembly while cellulose in the wall increased. Zygotes from this stage of development also possessed a glucanase which is released from a particulate fraction by Triton X and is capable of hydrolyzing native laminaran to glucose. The enzyme appears to be a exo-β-1,3-glucanase since glucose and not laminarabiose or other oligosaccharides were found in incubation mixtures of the enzyme and native ^{14}C-laminaran. Relatively large increases in respiration rates following fertilization of *Fucus* eggs have been reported (*cf.* ref. 21). Thus, zygotes possess a mechanism for laminaran breakdown which in turn could provide glucose for cellulose and other polymer syntheses, as well as for ATP formation. Evidence for the ability of certain brown algae to fix CO_2 into carbohydrates in the dark (16) may indicate a similar pathway operating in *Fucus* zygotes to provide carbon substrates for wall formation.

In this study, we have identified polysaccharides that are assembled into a new cell wall at specific times during early development in *Fucus*. This wall assembly is initiated around a wall-less egg cell in response to fertilization and proceeds synchronously in a large population of zygotes in the absence of growth and cell division. Subsequent reports will be concerned with the pathway(s) of cytoplasmic synthesis and transport, as well as the mechanism of extracellular assembly of each polymer into the wall structure.

Acknowledgments – We thank S. Johnson and A. Ley for their technical help in the preliminary stages of this work. In addition, the assistance and facilities made available by the staff of the Institute of Marine Biochemistry in Trondheim, Norway, is gratefully appreciated. Discussions

and collaboration at various stages of this work with A. Haug, W. Hogsett, B. Larsen, F. Loewus, and D. Medcalf have been extremely helpful.

LITERATURE CITED

1. Baardseth, E. 1966. Localization and structure of alginate gels. *In:* Proceedings of 5th International Seaweed Symposium. Pergamon Press, New York. pp. 19-28.
2. Boffey, S. A. and D. H. Northcote. 1975. Pectic synthesis during the wall regeneration of plasmolyzed tobacco leaf cells. Biochem. J. 150: 433-440.
3. Brawley, S. H., R. Wetherbee, and R. S. Quatrano. 1976. Fine structural studies of the gametes and embryo of *Fucus vesiculosus* L (Phaeophyta). II. The cytoplasm of the egg and young zygote. J. Cell Sci. 20: 255-271.
4. Crayton, M. A., E. Wilson, and R. S. Quatrano. 1974. Sulfation of fucoidan in *Fucus* embryos. II. Separation from initiation of polar growth. Dev. Biol. 39: 134-137.
5. Hanke, D. E. and D. H. Northcote. 1974. Cell wall formation by soybean callus protoplasts. J. Cell Sci. 14: 29-50.
6. Haug, A. and B. Larsen. 1962. Quantitative determination of the uronic acid composition of alginates. Acta Chem. Scand. 16: 1908-1918.
7. Haug, A., B. Larsen, and O. Smidsrød. 1974. Uronic acid sequence in alginate from different sources. Carbohydr. Res. 32: 217-225.
8. Hogsett, W. E. and R. S. Quatrano. 1975. Isolation of polysaccharides sulfated during early embryogenesis in *Fucus*. Plant Physiol. 55: 25-29.
9. Horine, R. K. and A. W. Ruesink. 1972. Cell wall regeneration around protoplasts isolated from *Convolvulus* tissue culture. Plant Physiol. 50: 438-445.
10. Larsen, B., A. Haug, and T. Painter. 1970. Sulfated polysaccharides in brown algae. III. The native state of fucoidan in *Ascophyllum nodosum* and *Fucus vesiculosus*. Acta Chem. Scand. 24: 3339-3352.
11. Levring, T. 1952. Remarks on the submicroscopical structure of eggs and spermatozoids of *Fucus* and related genera. Physiol. Plant 5: 528-539.
12. Ley, A. C. and R. S. Quatrano. 1973. Isolation and characterization of cell walls from *Fucus* embryos. Biol. Bull. 145: 446.
13. Mian, A. J. and E. Percival. 1973. Carbohydrates of the brown seaweeds *Himanthalia lorea, Bifurcaria bifurcata,* and *Padina pavonia*. Carbohydr. Res. 26: 133-146.
14. Moon, A. and M. Forman. 1972. A procedure for the isolation of cell walls of *Fucus furcatus* embryos. Protoplasma 75: 461-464.
15. Nečas, O. 1971. Cell wall synthesis in yeast protoplasts. Bacteriol. Rev. 35: 149-170.
16. Nisizawa, K., H. Akagawa, and T. Ikawa. 1971. Dark $^{14}CO_2$-fixation in brown algae. *In:* Proceeding of 7th International Seaweed Symposium. John Wiley and Sons, New York. pp. 532-536.
17. Novotny, A. M. and M. Forman. 1974. The relationship between changes in cell wall composition and the establishment of polarity in *Fucus* embryos. Dev. Biol. 40: 162-173.
18. Novotny, A. M. and M. Forman. 1975. The composition and development of cell walls of *Fucus* embryos. Planta 122: 67-78.
19. Preston, R. D. 1974. The Physical Biology of Plant Cell Walls. Chapman and Hall Ltd., London.
20. Quatrano, R. S. 1972. An ultrastructural study of the determined site of rhizoid formation in *Fucus* zygotes. Exp. Cell Res. 70: 1-12.
21. Quatrano, R. S. 1974. Developmental biology: development in marine organisms. *In:* R. N. Mariscal, ed., Experimental Marine Biology. Academic Press, New York. pp. 303-346.
22. Quatrano, R. S. and M. A. Crayton. 1973. Sulfation of fucoidan in *Fucus* embryos. I. Possible role in localization. Dev. Biol. 30: 29-41.
23. Seno, N., K. Anno, K. Kondo, S. Nagase, and S. Saito. 1970. Improved method for electrophoretic separation and rapid quantitation of isomeric chondroitin sulfates on cellulose acetate strips. Anal. Biochem. 37: 197-202.
24. Smidsrød, O. 1974. Molecular basis for some physical properties of alginates in the gel state. Faraday Discuss. Chem. Soc. 57: 263-274.
25. Stevens, P. T. 1975. Cell wall formation in Fucus zygotes: cellulose synthesis and deposition after fertilization. Ph.D. dissertation. Oregon State University.
26. Tsai, C. M. and W. Z. Hassid. 1971. Solubilization and separation of uridine diphospho-D-glucose:β-(1,4)-glucan and uridine diphospho-D-glucose:β-(1,3)-glucan glucosyltransferases from coleoptiles of *Avena sativa*. Plant Physiol. 47: 740-744.
27. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1964. Manometric Techniques, Ed. 4. Burgess Publishing Company, Minneapolis.
28. Updegraff, D. M. 1969. Semi-micro determination of cellulose in biological materials. Anal. Biochem. 32: 420-424.
29. Vreugdenhil, D., M. L. Dijkstra, and K. R. Libbenga. 1976. The ultrastructure of the cell wall of normal and apolar embryos of *Fucus vesiculosus*. Protoplasma. In press.