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Title: CELL WALL FORMATION IN FUCUS ZYGOTES: CELLULOSE SYNTHESIS AND DEPOSITION AFTER FERTILIZATION

Abstract approved Redacted for privacy
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Under defined cultural conditions, large populations of zygotes of the brown algae Fucus vesiculosus L. and F. distichus L. Powell synchronously form a cell wall shortly after fertilization. Intact cell walls, free of cytoplasm and maintaining shape properties of early stages of embryo development, have been isolated from these zygotes. Removing components of the wall soluble in 0.25 N HCl, 3% $Na_2CO_3$ and 10% KOH at 110° C does not affect the shape properties of these isolated walls. Chromatography of acid and enzymatic hydrolysates of the remaining cell wall material identified the component which maintains cellular shape as the β-1,4-glucan, cellulose. Neither β-1,3-linkages nor protein are detectable in this cellulose fraction. Cellulose is not detectable in eggs but is present in zygotes within 20 minutes following fertilization. Incorporation of radioactivity from

the precursors $NaH^{14}CO_3$ and $^3H$-glucose confirmed that at least a portion of this cellulose is newly synthesized after fertilization with the highest rate of synthesis occurring in this first 20 minute period. During the first 24 hours of development the greatest amount of cellulose accumulates during the first four hours after fertilization, and most, if not all, of this cellulose is found associated with the cell wall and not the cytoplasm. This evidence suggests that the assembly, and possibly the synthesis of cellulose, occurs at the plasmalemma or extracellularly. Use of specific inhibitors indicated that cellulose synthesis requires cellular energy and neither cellulose synthesis nor cellulose deposition is regulated by genetic controls at transcription or translation. The evidence is consistent with the hypothesis that cellulose-synthesizing enzymes are pre-formed in the egg and activated upon fertilization. A particulate β-1,3-glucanase which is solubilized by Triton-X was isolated from zygotes and shown to release only glucose from the storage β-1,3-glucan (laminarin) purified from eggs and zygotes. Breakdown of laminarin by this exo-β-1,3-glucanase may provide substrates used for cellulose synthesis at the time of cell wall formation. Cellulose synthesis and deposition into a completely new cell wall formed synchronously in a single-cell population of Fucus zygotes can now serve as a model system to study mechanisms and regulation of cell wall biogenesis.

# Cell Wall Formation in *Fucus* Zygotes: Cellulose Synthesis and Deposition After Fertilization

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# CELL WALL FORMATION IN FUCUS ZYGOTES: CELLULOSE SYNTHESIS AND DEPOSITION AFTER FERTILIZATION

## I. INTRODUCTION

### A. Overview of Problem

In both animals and plants shape plays an important role in determining the function and morphogenetic pattern of molecules, organelles, cells, tissues and organs. Changes in organismic shape begin to occur early in embryology. During early development in animals the movement of cells relative to one another during gastrulation is responsible for changes in the shape of the embryo and the initiation of histogenesis and organogenesis (Balinsky, 1970). In plants changes in morphology are based on the direction, distribution and duration of cellular growth (i.e., cell division and cell elongation) within tissues. This is clearly evident during early embryogenesis in the formation of cotyledons, the first morphological change of the globular dicot embryo (Maheswari, 1963; Steeves and Sussex, 1972). This stage of morphogenesis in angiosperms is analogous to gastrulation in animal embryogenesis except that the plant cells have a fixed position relative to their neighbors. Because of this, knowledge of the processes controlling deposition and alignment of the plant cell wall during cell growth is important for understanding the biochemical basis of cellular shape and subsequent tissue and organ form.

The wall of a plant cell is composed of a continuous matrix of polysaccharide material with varying amounts of protein (Muhlethaler, 1967; Lamport, 1965). Embedded in this matrix is a highly organized pattern of microfibrils that are composed of cellulose (Preston, 1964). Cellulose consists of chains of D-glucose units joined by β-1,4 glycosidic bonds, with a molecular weight of approximately 600,000 (Teng and Whistler, 1973). The molecule is believed to be in the conformation of a flat ribbon stabilized by intrachain hydrogen bonding (Preston, 1964). In the electron microscope cellulose appears to be arranged into microfibrillar structures (approximately 50-150 Å in diameter) which are often oriented in a direction specifically related to cell dimensions (Northcote, 1969b). An important factor in determining the conformational complexity of these microfibrils within the cell wall is the association between cellulose and other matrix polysaccharides.

The matrix polysaccharides of higher plants are the acidic polysaccharides, pectins, and the group of neutral polysaccharides called the hemicelluloses. Pectins are D-galacturonan backbones which are cross-linked by metal ions. The matrix polysaccharides of various divisions of algae include fucoidan, agar, carrageenan and alginic acid. The latter is found in the Phaeophyta and is functionally analogous to the pectic substances of higher plants because of its ability to bind ions in a specific manner (Wasserman, 1948) and its

uronic acid composition (Percival and McDowell, 1967).

Although the major fibrous support polysaccharide in higher plant walls is the β-1,4-glucan, cellulose, many algae have polysaccharides other than cellulose for their support component. A β-1,3-xylan provides the major structural polysaccharide in the algae Bryopsis and Caulerpa; a β-1,4-mannan is found in Acetabularia and Codium; Halicystis uses xylan and cellulose; and in Hydrodictyon, a mannan is found in combination with cellulose. Even in higher plants special support materials other than cellulose are sometimes found, such as the mannan in cell walls of certain seeds (Kirkwood, 1974).

Current approaches to the study of how these components are assembled into the cell wall include the following; ultrastructural studies of cell wall deposition in a variety of cells including the newly fertilized egg in certain angiosperms, regeneration of cell walls around protoplasts which have had a pre-existing cell wall removed by hydrolytic enzymes, and chemical characterization of the wall of mature plant cells. These approaches have led to further research aimed at answering the questions: What are the synthetic pathways of wall polysaccharides? What is the subcellular site of synthesis and assembly? What controls the orderly deposition of wall polysaccharides into a complex three-dimensional structure? Since the main structural component of most plant walls is cellulose, and the main concern of this research involves this component, subsequent

discussion will focus primarily on this macromolecule.

## B. Biogenesis of Cellulose

### 1. Synthetic Pathway

The general scheme for the synthesis of cellulose, a β-1,4-linked glucan polymer, appears to be:

$$\text{GTP} + \text{glucose-1-P} \xrightleftharpoons[\text{GDP-glucose pyrophosphorylase}]{} \text{GDP-glucose} + \text{PPi}$$

$$\text{GDP-glucose} + \text{acceptor} \xrightarrow[\text{transglycosylase}]{} \text{acceptor-}\beta\text{-1,4-glucose} + \text{GDP}$$

The cellulose-synthesizing enzyme system has a high degree of specificity for GDP-glucose as the sugar nucleoside precursor (Barber et al, 1964), but activity has also been obtained with UDP-glucose (Larsen and Brummond, 1974; Clark and Villemez, 1972). Delmer et al (1974) have shown that GDP-glucose serves as precursor for primary wall cellulose in cotton but that this activity disappears during secondary wall cellulose deposition. The primary acceptor substance in the complete reaction has not yet been identified due to inability to separate the transferase activity from the endogenous acceptor (Hassid, 1969). Larsen and Brummond (1974) reported an enhancement in cellulose synthesis when glucose, cellobiose or

methyl-β-D-glucopyranoside were added to reaction mixtures. The enzyme system is particulate and can be solubilized by digitonin (Tsai and Hassid, 1971; Larsen and Brummond, 1974). Its activity can be stimulated by addition of $Mg^{+2}$ (Hassid, 1969).

## 2. Site of Synthesis and Assembly

Two hypotheses have been advanced with respect to the site of cellulose synthesis: (i) extracellular, associated with the plasmalemma or in the cell wall itself; and (ii) intracellular, within specific organelles such as the Golgi and deposited outside the cell by exocytosis.

a. Extracellular. The first hypothesis is supported by observations of freeze-etched cells in which particles can be seen on the outer surface of the plasmalemma. From these particles, cellulose microfibrils appear to radiate (Northcote, 1969a; Northcote and Lewis, 1968). Also, in an autoradiographic study of sycamore cells pulsed with $^{3}H$-glucose, Wooding (1968) observed that labeled material was present between the plasma membrane and cell wall. None appeared over the Golgi bodies or any other organelle in the cytoplasm.

Ruiz-Herrera and Bartnicki-Garcia (1974) have recently demonstrated the in vitro synthesis of cell wall microfibrils by chitin synthetase isolated from Mucor rouxii. The synthetase activity is

initially found bound to a membrane fraction of cellular homogenates but is released from this fraction into the soluble supernatant when exposed to its substrate, UDP-N-acetylglucosamine, and an activator, N-acetyl-D-glucosamine. The fibrous material synthesized by this enzyme resembled chitin microfibrils of Mucor cell walls when viewed under the electron microscope. X-ray diffraction analyses proved that the product was identical to highly purified chitin. These findings demonstrate that although the synthetase is membrane-bound, it is released by the substrate and can form polymer in the absence of membrane. The implication is that assembly of the wall microfibrils can occur extracellularly, in the absence of other matrix polysaccharides. However, there is no evidence from their work that identifies the membrane component to which the synthetase is initially bound.

In vitro reassembly of two subunits of the cell wall of Chlamydomonas has been demonstrated by Hills (1973). Cell walls prepared from this alga dissociate in 8 M lithium chloride into two components with sedimentation velocities of 6.8 S and 9.3 S. If a solution of these subunits is dialysed against water to remove the lithium chloride, reassembly of the cell wall occurs. The reassembled wall components are indistinguishable from normal wall components in fine structure and when subjected to polyacrylamide gel electrophoresis. Reassembly of the subunits is dependent on the presence of an as yet unidentified nucleating agent found in the residue

after dissociation and centrifugation of walls in 8 M lithium chloride. The nucleating agent appears to initiate reassembly of subunits and is then incorporated into the reassembled cell wall. Although the final wall is capable of assembly extracellularly, individual polymers such as cellulose could have been synthesized within the cell.

b. Intracellular. Support for the involvement of the Golgi in cellulose synthesis has been provided by several morphological and biochemical studies. Cell wall fragments of the marine alga Pleurochrysis have been studied extensively and shown to consist of a cellulosic glycoprotein (Herth et al, 1972). Wall fragments ("scales") of this alga can be collected from zoospore mother-cell dissolution during zoospore formation. The scales resist alkaline hydrolysis and when completely hydrolyzed with acid, yield primarily glucose and traces of galactose as determined by thin-layer and gas chromatography (Brown et al, 1969; Herth et al, 1972). X-ray diffraction patterns of the scale material compared well with those of cotton cellulose, suggesting that cellulose is the main crystalline component of the scales. The peptide component is covalently linked to this cellulosic polysaccharide and remains associated with it through alkaline purification treatments. The cellulose : protein ratio (w/w) of alkali-purified wall fragments is approximately 2:1.

Brown et al (1969) have presented electron and light microscopic evidence that suggests that Golgi plays a role in the synthesis of these

scales. There appears to be only one Golgi apparatus per cell and it has both compact and distended cisternae, the latter always oriented toward the surface of the cell. The scales are observed within the distended cisternae in the same form as found in the wall. It should be noted, however, that the cellulosic scales that form a plated armor around this alga represent an architecturally different type of envelope from the typical cell wall of the vast majority of fungi and green plants. In the latter, the wall is a continuous microfibrillar structure surrounding the entire cell.

When pea stem segments were pulsed for 60 minutes with $^{3}$H-glucose, and then chased for 30 minutes in "cold" glucose, autoradiographs of the cells demonstrated label over Golgi vesicles and in the wall (Ray, 1967). However, a chemical analysis of this radioactive material indicated that 25% occurred in hemicellulose. Therefore, it is not clear whether labeled precursors seen over Golgi become incorporated into the cellulose or hemicellulose fraction of the wall.

Engels (1974) has shown that the contents of Golgi vesicles from germinating Petunia pollen gave similar x-ray diffraction patterns to those of the cellulose in the wall. Golgi vesicles have been observed to migrate to and fuse with the plasmalemma in various plant cells (Sassen, 1964; Van der Woude et al, 1971). Thus, cellulose may be synthesized intracellularly in the Golgi and assembled into

microfibrils extracellularly. From this point of view cellulose synthesis within the Golgi vesicles need not be considered contrary to synthesis at the site of the plasma membrane (Engels, 1974).

3. Deposition into Cell Wall

a. Relationship to Microtubule Orientation. How is cellulose deposited in an orderly pattern in regions of wall growth? Ever since the discovery by Ledbetter and Porter (1963) of microtubules in the peripheral cytoplasm of plant cells which were oriented parallel to the cellulose microfibrils of the wall, there has been an interest in their possible role in the control of cell wall deposition. Subsequent investigations have established the mutual alignment between microtubules and cellulose microfibrils occurs in higher plants (Newcomb, 1969; Pickett-Heaps, 1967) as well as in some algae (Pickett-Heaps, 1972). Removal of microtubules with the alkaloid colchicine leads to disruption of the cellulose microfibril pattern (Hepler and Fosket, 1971; Pickett-Heaps, 1967). A current hypothesis suggests that microtubules play a role in aligning cellulose microfibrils, possibly by orienting the flow of Golgi vesicles containing the cell wall precursors or cell wall synthesizing enzymes.

b. Wall Assembly in Protoplasts. Although much information has been obtained from these studies, all of them involve addition of wall material to an already established three-dimensional structure.

What is known about the initial assembly of a cell wall from a wall-less progenitor cell? Prior to fertilization in several species of higher plants for which detailed microscopic observations are available (Schulz and Jensen, 1968; Cocucci and Jensen, 1969; Diboll, 1968), a cell wall is present only over the micropylar end of the egg. However, a complete cell wall forms around the zygote after fertilization. In early zygotes of Quercus, Mogensen (1972) observed that the cell wall developed from the micropylar end and appeared to be laid down unevenly at first, discontinuous in places, but later became evenly thickened around the entire zygote. Only cytological evidence is now available in these systems because of the difficulty in isolating large numbers of unfertilized eggs for biochemical studies.

Due to the difficulty of experimentally manipulating cell wall deposition in higher plant zygotes, isolated protoplasts represent the best model system for studying cell wall assembly. Protoplasts are obtained by subjecting suspension cultures of somatic cells to a mixture of hydrolases which remove the cell walls. The literature on cell wall regeneration in these protoplasts is extensive (c.f. Cocking, 1972), but the salient points can be summarized as follows:

(i) Regeneration of walls appears to occur almost immediately after protoplasts are removed from the hydrolases and placed in nutrient medium containing an osmotic stabilizer.

(ii) Infoldings of the plasmalemma appear within three hours and electron-dense material is accumulated near the plasmalemma.

(iii) A wall forms, as determined by the ability of the protoplast to plasmolyze away from the cell wall, after about three days.

(iv) After five days of culture, fibrils, which are probably microfibrils, are visible by freeze-etching of the outer cell wall surface.

Analysis after feeding radioactive glucose to soybean protoplasts undergoing wall regeneration showed only a small proportion (1-5%) of the label was incorporated into polymers over a 40-hour cell wall regeneration period (Hanke and Northcote, 1974). However, since the starch grains present in the protoplasts were observed to undergo reduction in size and number during the period of wall regeneration, the proportion of polymeric material which could be labeled from tracer may have been reduced by a contribution of unlabeled sugars from the breakdown of endogenous starch. Of particular interest was the finding that the regenerating walls were completely devoid of radioactive pectin. This component was not incorporated into the regenerating wall but rather excreted into the medium. Thus, during the first 40 hours of wall regeneration, at least, a normal cell wall component is extruded from the protoplasts rather than deposited into the extracellular structure.

Use of isolated protoplasts for studying cell wall regeneration presents several problems:

(i) Doubt exists as to whether the small discontinuous amounts of material seen at the plasmalemma surface after 24 hours represent new wall or fragments of the original tissue wall (Burgess and Fleming, 1974).

(ii) Willison and Cocking (1972) report that during cell wall regeneration of tomato-fruit protoplasts "there is much variation between individual protoplasts in the nature of the wall formed and the timing of development." Lack of homogeneous cell types in the cultures from which protoplasts are prepared and lack of synchrony in the cell wall regeneration process are major obstacles to biochemical analyses.

(iii) A native component of higher plant cell walls, pectin, is actually extruded from protoplasts into the culture medium during early wall regeneration (Burgess and Fleming, 1974).

Additional information has been provided by studies of fungi and algae. Yeast protoplasts, isolated by autolysis or digestion of the wall with snail enzymes, regenerate only the fibrillar component of normal cell walls when cultured in liquid medium (Nečas, 1971). They regenerate both the fibrillar network and the amorphous matrix of normal cell walls when embedded in a gel medium. Thus, the biosynthesis of fibrils can be studied separately from the synthesis of matrix.

The structural element of the regenerating wall in liquid culture is a microfibril containing primarily β-1,3-glucan. If glucose in the nutrient medium is replaced with deoxyglucose, which interferes with glucan synthesis, no microfibrils form on the protoplasts (Nečas, 1971). Cycloheximide does not affect fibril formation but it does inhibit the synthesis of wall matrix (Farkas et al, 1970). These studies, coupled with the observation that in vitro reaggregation of fibrillar elements can be obtained from dissolved glucan, have led to the suggestion (Nečas, 1971) that the protoplasts secrete barely soluble glucan or supramolecular aggregates of glucan which form a concentration gradient around the protoplasts. The microfibrils could then originate through self-assembly, or crystallization, of these accumulated elements. A similar suggestion, based on in vivo studies of the site of cellulose synthesis, has been made by Engels (1974) concerning higher plant cell formation, i.e., that glucan polymers may be synthesized intracellularly and assembled into microfibrils extracellularly (see Section B2b).

## C. Experimental System and Purpose of Study

Although cell wall material, free of cytoplasmic debris, can be isolated for study from a variety of plant cells (Herth et al, 1972; Hills, 1973; Moon and Forman, 1973), few if any studies report isolation of walls in intact form, i.e., maintaining morphological

characteristics of a particular stage of development. Many of the experimental systems used for studies of cell wall formation involve multicellular tissues where the forces exerted on wall deposition by surrounding cells are difficult to assess. Also, in these experimental systems cell wall formation occurs by extension of a pre-existing wall at the time of cell division. The disadvantages of using isolated protoplasts for studying cell wall formation have been discussed previously.

Zygotes of the brown alga Fucus provide a unique system for the study of cell wall deposition around a wall-less progenitor cell. Like angiosperms, eggs of this alga have no cell wall (Levring, 1952) but within 30-60 minutes after fertilization a cell wall is present around the young zygotes. Time of fertilization can be controlled within ± 15 minutes in F. vesiculosus (dioecious) and ± 30 minutes in F. distichus (monoecious), and the population develops synchronously in a defined inorganic sea water medium under controlled laboratory conditions (Quatrano, 1974). The cell wall increases several micrometers in thickness during the first 16 hours after fertilization, but the cell does not divide and there is no increase in size or change in shape of the zygote during this period. Between 16 and 20 hours after fertilization a characteristic change in shape (rhizoid formation) of the zygote occurs which is not dependent on cell division or spindle apparatus (Quatrano, 1973). The first cell division occurs between

20 and 24 hours.

Not only can one study new cell wall deposition in this system, but also cell wall extension when cell growth occurs at a specific site in the zygote wall at 16 hours, i.e., rhizoid formation. Since the shape of this new wall outgrowth is unique, environmental factors and polymers in the wall responsible for initiating and stabilizing this cellular morphogenesis can be experimentally analyzed. Hence, a natural trigger (fertilization) begins a developmental program to deposit a cell wall around a wall-less egg without concomitant cell growth or cell division. This wall is deposited around every cell in large populations of synchronously developing single cells under defined culture conditions. In addition, the pattern of growth and morphogenesis observed during early Fucus embryogenesis is similar to that of many other plant groups, including angiosperms (c.f. Maheswari, 1963; Wardlaw, 1968).

Using Fucus as an experimental system to study cell wall assembly, the major goals of this research are:

(i) To determine what structural component, if any, of the wall network is responsible for maintaining cell shape.

(ii) To chemically characterize this structural polysaccharide component of the cell wall by hydrolysis (chemical and enzymatic). Evidence will be presented that cellulose is the component of the cell wall that maintains cell shape.

(iii) To study the rate of synthesis and deposition of cellulose into the cell wall at various stages after fertilization by labeling with radioactive precursors.

(iv) To determine the level of genetic control of cellulose synthesis and deposition into the wall by using inhibitors that block gene expression.

(v) To determine what storage polysaccharide, if any, is metabolized to provide the energy and carbon substrates for cellulose assembly into the new cell wall following fertilization.

# II. MATERIALS AND METHODS

## A. Preparation of Biological Material

Receptacles of Fucus distichus L. Powell (collected at Yaquina Head, Newport, Oregon) were washed several times with cold tap water and blotted dry between paper towels. The receptacles were placed in shallow trays and covered with Instant Ocean (Aquarium Systems, Wickliffe, Ohio) or the following sea water mixture: 0.45 M NaCl, 0.01 M KCl, 0.009 M $CaCl_2$, 0.016 M $MgSO_4$ and 0.035 M $MgCl_2$ (Kinoshita, 1971). Receptacles were then placed in a lighted incubator (600 ft.c.) at 15°C for gamete release. Since no difference in response was observed between these mixtures, they were utilized interchangeably. Hereafter, both mixtures will be referred to as artificial sea water (ASW).

F. distichus is monoecious and fertilization can occur during, or very shortly after shedding of the gametes from the receptacles. This shedding usually takes place in the light within 60 minutes after the inductive treatment described above. After shedding, the receptacles were removed and the zygote suspension poured through a 102 μm Nitex nylon mesh (Tobler, Ernst and Traber, Inc., New York, NY). This mesh size allowed the zygotes to pass through but retained the larger debris, e.g., intact oogonia, frond fragments, etc. Uniform cell suspensions of the filtered zygotes were pipetted into

100 X 15 mm glass petri dishes and returned to a lighted growth chamber at 15°C for further incubation and experimental manipulation. Development occurred for various periods of time up to 48 hours depending on the individual experiment.

Receptacles of F. vesiculosus L. (collected at Manomet, Massachusetts), were obtained from the Marine Biology Laboratory, Woods Hole, Massachusetts. They were treated similarly except that since this species is dioecious, male and female receptacles were allowed to discharge gametes into separate containers so that sperm and eggs could be collected separately. Fertilization usually occurs within 15 minutes after mixing suspension of sperm and eggs. Thirty minutes after mixing, uniform cell suspensions of zygotes were pipetted into petri dishes and incubated for various periods up to 48 hours.

Eggs of F. vesiculosus were labeled during oogenesis by incubating washed female receptacles in ASW containing $NaH^{14}CO_3$ (1 μCi/ml ASW). Receptacles were placed in a lighted incubator (1000 ft. c.) for 36-48 hours 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 stored 7-10 days at 4°C in the dark. At the end of this storage period gametes were shed into ASW according to the procedure previously described.

To label polysaccharides after gametes were shed from receptacles, uniform zygote suspensions were incubated for various times in the presence of $NaH^{14}CO_3$ (0.75-1.0 μCi/ml ASW) or $^3$H-D-glucose (2 μCi/ml ASW).

To test the effect of various metabolic inhibitors on cell wall formation, appropriate concentrations of inhibitors were added to cultures of zygotes during the incubation period. Specified concentration ranges of the following inhibitors were tested: cycloheximide (Nutritional Biochemicals), 0.1-100 μg/ml ASW; colchicine (Nutritional Biochemicals), 0.5-100 mg/ml; cytocholasin B (Imperial Chemical Industries, Cheshire, England), 0.5-100 μg/ml; deoxyglucose (Sigma), 0.5-100 mM; coumarin (Eastman), $10^{-5}$-$10^{-3}$ M; dichlorobenzylnitrile (Eastman), $10^{-5}$-$10^{-3}$ M; and carbonylcyanide p-trifluoromethoxy phenylhydrazone (gift from M. Gibbs, Brandeis University), $10^{-6}$-$10^{-5}$ M.

All were aqueous solutions, stored at 4°C, except cytocholasin B which was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. Prior to use, the stock solution of DMSO was diluted with distilled water and then added to ASW. The final concentration of DMSO in cultures was always less than 1%, which had no effect on embryo development (Quatrano, 1973; Nelson and Jaffe, 1973; Novotny and Forman, 1974).

## B. Cell Wall Purification

Embryos were homogenized in distilled water in a power-driven Kontes Duall sintered glass homogenizer for 5 minutes and the volume of each sample brought to 10 ml. A 1 ml aliquot of the homogenate (whole embryo fraction) was removed, made to 70% (v/v) ethanol and stored at -20° C until further use. Cell walls and wall debris were collected from the remaining homogenized sample by centrifugation at low speed (100 g) on an International table-top centrifuge. The pellet was washed three times with 10 ml of distilled water and the washings pooled and saved. After resuspension in 10 ml of distilled water the washed pellet was sonicated for 30 seconds every minute in a Cell Disruptor (Branson Model W185) at a setting of 70 watts for a total of 10 minutes. Cell walls and wall fragments were then collected, washed five times with 10 ml of distilled water each time, and saved for further analysis (cell wall fraction). The washings were pooled and combined with the washings collected previously. The total pooled washings (80 ml) were strained through Miracloth to remove any contaminating cell wall fragments and were then made 70% with cold ethanol, centrifuged at 10,000 rpm for 10 minutes and the pellet stored at -20° C in fresh 70% ethanol (cytoplasmic fraction).

## C. Cellulose and Laminarin Extraction

Embryos were homogenized in 80% ethanol in a Duall sintered glass homogenizer driven by an electric motor for 5 minutes. The homogenate was centrifuged (6,000 rpm/10 minutes) and the residue was then sequentially extracted, according to the procedures of Mian and Percival (1973), by stirring in the following solutions: 2% (w/v) aqueous $CaCl_2$ for 1 hour at room temperature, for removal of laminarin and fucans; 0.25 N HCl for 30 minutes at room temperature, for extraction of fucans; 3% (w/v) aqueous $Na_2CO_3$ for 1 hour at 55°C, for removal of alginic acid and fucans; 10% (w/v) KOH at 110°C for 10 minutes, for removal of less soluble heteropolysaccharides. The final extraction with KOH was repeated with fresh solution three times and the remaining residue washed several times with water. The washed pellet (cellulose) was resuspended in a known volume of water and aliquots of this suspension analyzed for glucose content and radioactivity. For some experiments, cellulose was lyophilized with a Virtis dry ice freeze-dryer (Model 10-117).

To determine only the cellulose content of certain stage embryos or embryo fractions (e.g., cell walls, washings), the ethanol insoluble pellets were extracted directly with 10% KOH as described above. Glucose content and radioactivity determinations were then made on cellulose.

The procedure of Mian and Percival (1973) was used to extract laminarin. The fraction solubilized by $CaCl_2$ was dialyzed overnight against deionized water, and concentrated to a volume of 2 ml on a rotary-type evaporator. This fraction was applied to a cellulose (DE-52) microgranular, Whatman ion exchange column (1 X 15 cm) which had been equilibrated with 0.5 M KCl. The column was eluted with deionized water (1.5 bed volumes). The eluant was dialyzed overnight against deionized water, 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 rpm for 10 minutes at 4°C. The white, ethanol-insoluble precipitate was resuspended in 2 ml of water for determination of glucose and radioactivity.

## D. Methods of Chromatography and Hydrolysis

Embryos were incubated continuously in ASW cultures containing $NaH^{14}CO_3$ (1 μCi/ml) for 24 to 48 hours after fertilization. KOH-insoluble material (cellulose), extracted from these embryos according to procedures previously described, was suspended in 0.2 ml of 75% $H_2SO_4$ for 60 minutes at room temperature, then diluted 23:1 with water and autoclaved 60 minutes. The hydrolyzed sample was made neutral by adding a saturated solution of $CaCO_3$ and the precipitate removed by centrifugation. The supernatant was treated with

Amberlite IR 120 ($H^+$) ion exchange resin and the hydrolysate concentrated by evaporation. One hundred microliters of the hydrolysate were spotted along with glucose standards on 20 X 20 cm glass plates coated with a 250 μm layer of silica gel G and chromatographed in two different solvent systems: (1) n-butanol:glacial acetic acid:ethanol: water (9:6:3:1) for 2.5 hours; (2) acetone:water (90:10) for 60 minutes (Stahl, 1969, p. 833).

Laminarin, i.e., that fraction of the calcium chloride extract which eluted from a DE-52-cellulose column with water, was made 1 N with HCl and hydrolyzed by refluxing at 100°C for 3 hours.

Hydrolysates of both cellulose and laminarin were subjected to descending paper chromatography using Whatman 3 MM paper in solvent system (3) consisting of n-butanol:glacial acetic acid:water (5:1:2) (Stahl, 1969, p. 834) for 16 hours.

The labeled hydrolysates were scraped in 2-cm widths from the plates for detection of radioactivity by liquid scintillation counting. The glucose standard on the plates was sprayed with anisaldehyde reagent as described by Stahl (1969, p. 857). Unlabeled cellulose and laminarin hydrolysates were also chromatographed and sprayed with anisaldehyde or alkaline silver nitrate (Stahl, 1969, p. 889). Various sugars and uronic acids were also used as standards and their location on developed plates or paper were detected by spraying with

naphthoresorcinol or alkaline silver nitrate reagents (Stahl, 1969, pp. 857 and 888).

For gas chromatography trimethylsilyl derivatives of the dried hydrolysate were prepared according to Sweeley, et al (1963). These were injected into a Packard 417 Becker Gas Chromatograph containing a column filled with 3% OV-1 on Gas Chrom Q, 60-80 mesh. The column was maintained at 185° C with the injection port temperature at 235° C and the attenuation set at 8. The carrier gas was $N_2$ and the solvent utilized was heptane. The gas chromatographic analyses were performed at the Marine Biology Laboratory, Woods Hole, Mass.

Descending paper chromatography was used to resolve enzyme hydrolysates of the $^{14}C$-labeled alkali-insoluble fraction of embryos. One hundred microliters of the enzyme reaction mixtures were spotted on Whatman 3 MM paper along with 10 µl of a 10 mg/ml solution of the following standards: glucose, cellobiose and laminaribiose (obtained from E.T. Reese, U.S. Army Natick Laboratories, Natick, Mass.). The chromatograms were developed in solvent system (4) consisting of n-propanol:ethyl acetate:water (7:1:2) for 40 hours according to the method of Tsai and Hassid (1971). The dried paper chromatograms were cut into 2 X 3 cm strips and the radioactivity on the paper strips was counted in a liquid scintillation counter. Standards were detected by dipping the chromatograms into silver nitrate-sodium hydroxide stain (Stahl, 1969, p. 889).

## E. Enzyme Hydrolysis

To determine the nature of the glycosidic linkage in the alkali-insoluble material of embryos and dimer released by the use of endo-enzymes was identified. Purified endo-β-1,4-glucanase and endo-β-1,3-glucanase were obtained from E.T. Reese, U.S. Army Natick Laboratories, Natick, Mass. Alkali-insoluble material was extracted as previously described from embryos labeled with $^{14}C$ for 24 hours after fertilization. Six milligrams (dry weight) of alkali-insoluble material was resuspended in 2 ml of water. The insoluble material contained 22 cpm of $^{14}C$ per μg of glucose as determined by assaying an aliquot with anthrone reagent and counting in a liquid scintillation counter. Two hundred microliters of the 2 ml suspension of alkali-insoluble material (containing 8,686 cpm of $^{14}C$) was incubated with endo-β-1,3 or endo-β-1,4-glucanase (2 mg per ml of reaction mixture) in sodium acetate buffer at pH 4.8 for 20 hours at 50° C. The reaction mixtures contained excess substrate to insure maximal release of labeled products. The total volume of reaction mixture was 0.5 ml. The reactions were terminated by centrifuging the incubation mixtures to remove unhydrolyzed substrate and the supernatants were stored at -20° C until paper chromatography could be performed to determine the products of hydrolysis. Aliquots of the reaction mixtures were counted in a liquid scintillation counter after centrifugation to

determine the percentage of counts released by endo-enzymes during the incubation. Endo-β-1,4-glucanase released 40.8% of the $^{14}C$ counts and endo-β-1,3-glucanase released 6.7% of the counts during the incubation period.

Incubation with endo-β-1,3-glucanase was also performed on laminarin. Reaction mixtures and incubation procedures were identical to those described above except that 200 µl of aqueous laminarin extract was substituted for KOH-insoluble substrate.

## F. Radioactivity Determinations

$NaH^{14}CO_3$ and D-glucose-2-$^{3}H$ were obtained from Amersham/Searle and had specific activities of 59.7 mCi/mmol and 500 mCi/mmol, respectively. Aliquots (100 µl) of soluble radioactive polysaccharides as well as suspensions of alkali-insoluble material from chemical fractionation were applied to Whatman 3MM filter discs and air dried. The sample filters were placed in 5 ml of Omnifluor (New England Nuclear) and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 2425) which operated at 88% efficiency for $^{14}C$ and 60% efficiency for $^{3}H$.

Scrapings from silica gel G chromatographic separations as well as paper strips from descending paper chromatographic separations were added directly to Omnifluor and counted. Samples were counted for periods sufficient to insure that the maximum counting

error was less than 1% standard deviation. All cpm reported were adjusted for background and corrected for quenching by use of an external standard.

## G. Methods of Assay

For determination of protein in the alkali-insoluble (cellulose) fraction of embryos, 5 mg of lyophilized cellulose, extracted according to procedures previously described, was hydrolyzed in 2 ml of 6 N HCl in sealed tubes for 22 hr at 110° C (Blackburn, 1968, p. 21). After hydrolysis, HCl was removed by evaporation on a rotary-type evaporator and the residue resuspended in 5 ml of distilled water. Bovine serum albumin (BSA) was acid-hydrolyzed similarly for use as a standard.

The ninhydrin assay for determination of protein was performed according to the method of Moore (1968). Ninhydrin solution was obtained as a gift from Dr. Robert Becker, Oregon State University, and contained 80 g ninhydrin and 2.5 g hydrindantin in 1 l of dimethyl sulfoxide (DMSO) and 1 l of 4 M lithium acetate buffer, pH 5.2. Varying concentrations of hydrolyzed BSA and cellulose, in a final volume of 0.6 ml at pH 5.2, were incubated with 0.5 ml ninhydrin solution for 90 minutes at room temperature. Absorbance at 570 nm was then determined with a Beckman DB Spectrophotometer. A minimum of 4 μg of hydrolyzed BSA was detectable by this method.

Protein in the alkali-insoluble fraction of embryos was also determined by the procedure of Lowry et al (1951) both before and after hydrolysis with 6 N HCl.

The anthrone assay, modified from Whistler and Wolfrom (1962, p. 390), was performed to determine the glucose content of the cellulose and laminarin fractions of embryos. Reaction mixtures contained aliquots of an aqueous suspension of the polysaccharide in 1.5 ml distilled water, 0.5 ml of 2% anthrone (Eastman) in ethyl acetate (w/v) and 6 ml concentrated $H_2SO_4$. These were shaken vigorously and allowed to incubate at room temperature for 30 minutes. An aqueous solution of glucose (200 μg/ml) was used as a standard. Absorbance at 625 nm was determined on a Spectronic 20 colorimeter. A minimum of 5 μg of glucose was detectable by this assay.

## H. Glucanase Preparation and Measurement of Activity

Zygotes washed with ASW were homogenized in 0.1 M citrate-phosphate buffer (pH 7.0) using 75-105 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 minutes. 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 milliliter of enzyme suspension. This suspension was incubated for

30 minutes and then centrifuged at 17,000 g for 10 minutes. The supernatant was dialyzed against 2 l of 0.01 M sodium acetate buffer (pH 4.8) for two hours and used as a crude enzyme extract. All of the above procedures were done at 4°C.

Activity of the crude glucosidase was measured by ability to hydrolyze various substrates. Laminarin (K and K Laboratories, Plainview, New York) and carboxymethylcellulose (Hercules Powder Company, Wilmington, Delaware) stocks were prepared at a concentration of 10 mg/ml in 0.1 M sodium acetate buffer (pH 4.8). $^{14}$C-labeled laminarin and $^{14}$C-labeled cellulose, extracted from Fucus embryos according to procedures described in Section IIC, and suspended in sodium acetate buffer (pH 4.8), were also used as substrates. When utilizing $^{14}$C-cellulose and $^{14}$C-laminarin as substrates, each reaction mixture contained 0.2 ml of labeled substrate (from 1,000 cpm to 4,500 cpm) in addition to 0.3 ml of crude enzyme extract. Incubation was at 37°C for various times up to 24 hours.

Hydrolysis of unlabeled substrates was measured by the presence of reducing sugars as determined by the Park and Johnson method (c.f. Umbreit et al, 1964, p. 209), using D-glucose as a standard. Hydrolysis of $^{14}$C-cellulose was determined by filtering the reaction mixtures immediately after incubation through a Whatman glass fiber (GF/A) filter and counting the filtrate in 10 ml of Aquasol and the filter in 5 ml of Omniflour. Hydrolysis of $^{14}$C-laminarin was

determined by subjecting reaction mixtures immediately after incubation to descending paper chromatography in solvent system (4) as described previously (Section IID). Radioactivity on the paper chromatograms was determined as described in Section IID.

# III. RESULTS

## A. Cell Wall Purification

The approach used to identify that component of the cell wall which maintains the shape or form of a plant cell was to isolate cell walls free of cytoplasm and possessing shape characteristics of a particular stage of development. By the sequential removal of chemically defined polymers from these isolated cell walls, along with microscopic examination of the extracted cell wall, the identification of those components forming the basis of cell shape could be ascertained.

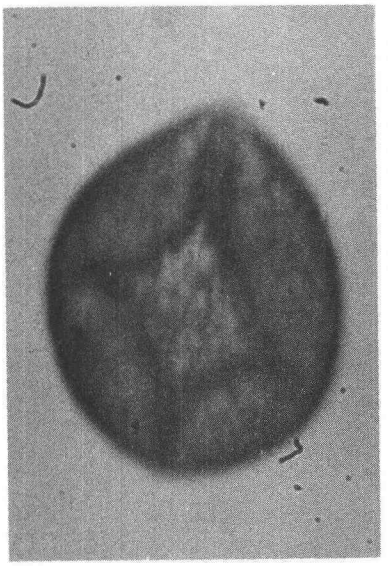
A cell wall is not observed when young zygotes of F. distichus are plasmolyzed with 3 M NaCl (Figure 1), or when eggs of F. vesiculosus are viewed with the electron microscope (Quatrano, 1968a; Brawley et al, 1974). Within four hours after fertilization, plasmolyzed zygotes clearly show a cell wall (Figure 2), as do electron micrographs of zygotes from F. distichus and F. vesiculosus (Quatrano, 1968a; Brawley et al, 1974). Deposition of a cell wall is also confirmed by the ability of 1-4 hour zygotes to rotate plane polarized light, resulting in a birefringent cell wall (Figure 3). This property is indicative of the presence of a highly ordered polymer(s). Birefringence is not evident in eggs or zygotes observed immediately after fertilization. Therefore, during the first four hours of

Figure 1. Zygote of F. distichus plasmolyzed with 3 M NaCl 15 minutes after fertilization. Note lack of a cell wall. (X600)

Figure 2. Zygote of F. distichus plasmolyzed with 3M NaCl four hours after fertilization showing a cell wall. (X600)

Figure 3. Zygote of F. vesiculosus four hours after fertilization showing a birefringent cell wall under plane-polarized light. (X600)

Figure 4. Isolated cell walls of F. distichus under dark field illumination. (X140)



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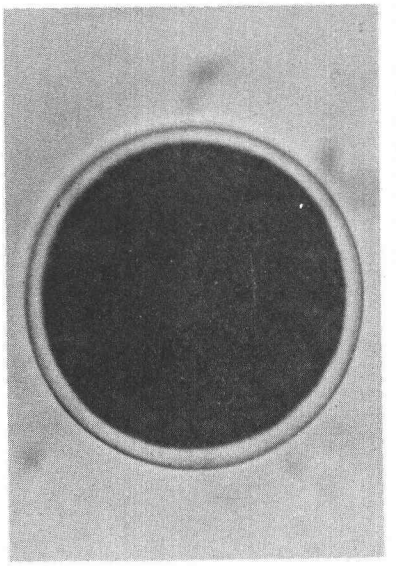

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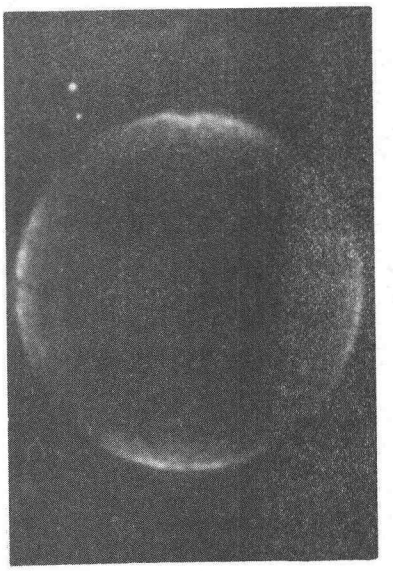

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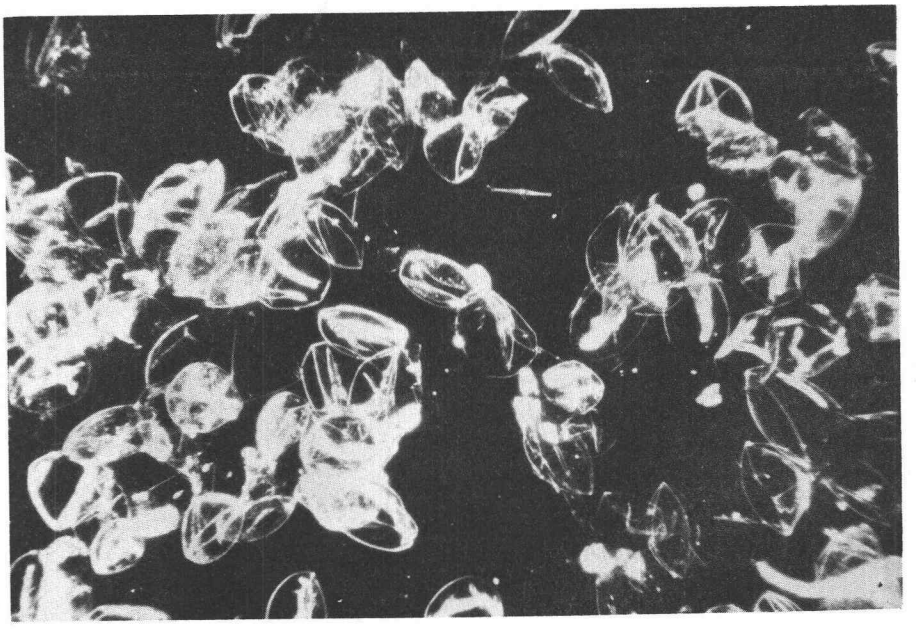


4

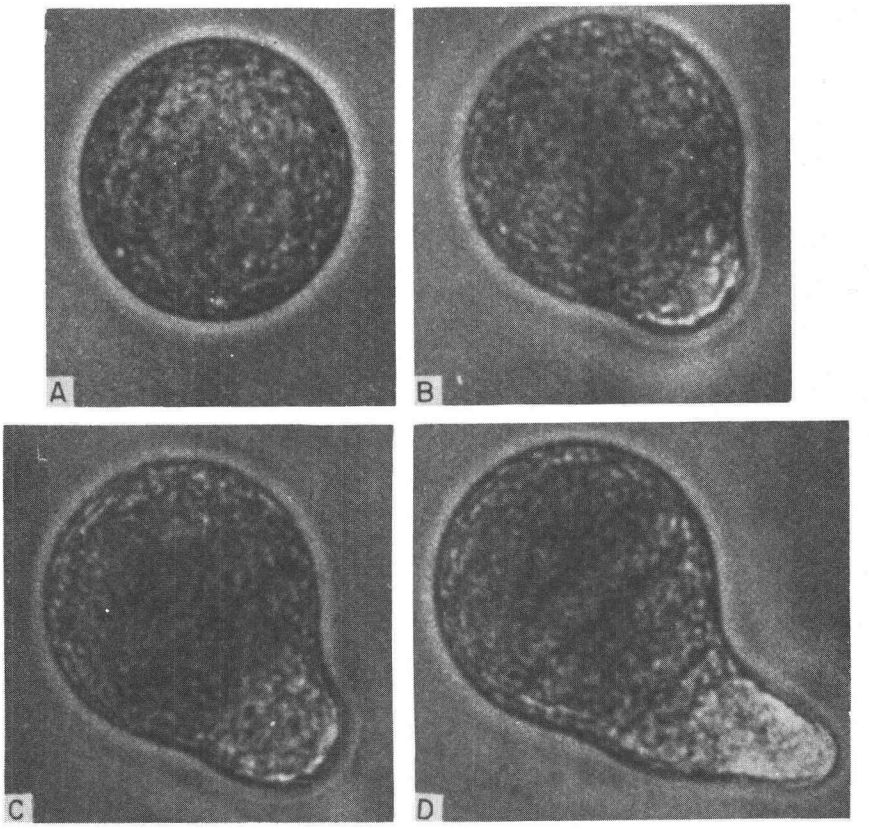
embryogenesis, a highly ordered structure is deposited on the surface of the Fucus egg in response to fertilization.

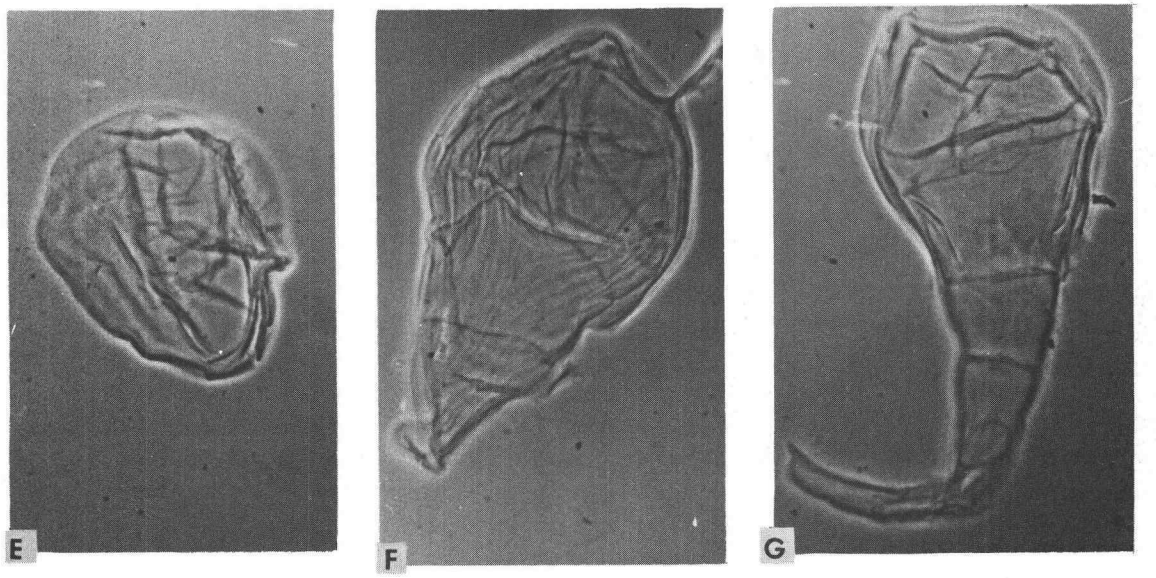
A method for obtaining intact cell walls of F. distichus embryos was developed. Cytoplasm is removed from cell walls by mechanical disruption, sonication and repeated homogenizations in distilled water (see Section IIIB). Light microscope observations of cell wall preparations purified in this manner showed them to be free of cytoplasmic contamination when observed under dark field and phase optics (Figure 4). To quantitate the amount of cytoplasmic contamination, $^{3}$H-fucose ($2.22 \times 10^{7}$ cpm) and fucoidan labeled with $^{35}$S ($1.6 \times 10^{6}$ cpm) were added to the initial cellular homogenate. After purification of walls by the method developed, only 0.013% of the $^{3}$H-fucose and 0.2% of the $^{35}$S-fucoidan originally added remained associated with the cell walls.

Cell walls obtained from embryos during the first four hours after fertilization, fragmented during the purification procedure and did not retain the shape of the embryo. Cell walls isolated four hours following fertilization remain intact (Figure 5E-G), and over 75% of the population maintain the structural characteristics indicative of a particular developmental stage (Figure 5A-D). This evidence coupled with birefringence measurements, indicates a stable cell wall structure is assembled during the first four hours after fertilization.

Figure 5A-D. Embryo development in Fucus. The spherical zygote (A) forms a rhizoid at about 16 hours after fertilization (B) which is partitioned from the rest of the cell by the first division at about 22 hours (C) and the second division at about 26 hours. (X500)

Figure 5E-G. Cell walls isolated from Fucus embryos at 4 hours (E), 24 hours (F) and 36 hours (G) after fertilization. Note that the structural characteristics of embryonic development are retained in the isolated walls (compare with A-D). These structural characteristics are retained even after isolated walls are treated with hot alkali. (X500)


A
B
C
D


E
F
G

Ley and Quatrano (1973), using a similar but much more drastic purification procedure, showed by chemical analyses and staining properties that important structural polymers of the in vivo cell wall are retained in isolated cell walls of F. vesiculosus embryos. Cell walls of F. distichus were subjected to chemical extraction using dilute acid, sodium carbonate and strong alkali to release well-characterized polysaccharide fractions (Mian and Percival, 1973; Hogsett and Quatrano, 1975). The shape characteristics of the isolated cell walls were not destroyed by any of these treatments. The structure of embryonic cell walls isolated from 4-24 hour embryos remains intact even after treatment with 10% KOH at 110°C and is identical in appearance to isolated cell walls prior to alkali treatment when viewed with the light microscope (see Figure 5E-G). The birefringent property of these treated walls is also retained. Further treatment of alkali-extracted cell walls with urea (8 M) and guanidinium chloride (2 M) did not affect their shape properties, suggesting that hydrogen-bonding is not critical for the specific form of these cell walls. Since intact cell walls characteristic of the shape of zygotes during early development can be obtained after hot-alkali treatment, the component of the cell wall which is present at four hours, stable to hot-alkali and possessing birefringent properties, appears likely to play a major role in shape-determining events during embryogenesis.

This alkali-insoluble fraction of whole embryos of F. distichus increases rapidly after fertilization (Figure 6). Only trace amounts of this material is present at the earliest time zygotes of F. distichus can be collected (30 minutes). Analyses of the dioecious species, F. vesiculosus, from which eggs can be obtained free of sperm, showed no detectable alkali-insoluble material in unfertilized eggs (see Section IIIC). Although the increase continues until the time of the first cell division (about 22 hours), it is particularly rapid during the first four hours following fertilization.

Since these increases are measured in whole embryos, do they represent deposition of the alkali-insoluble material in the cell wall? Is the majority of alkali-insoluble material found in the cytoplasm, or is it assembled directly into the wall from outside the plasma membrane?

Figure 6 demonstrates that the alkali-insoluble material in the cytoplasm remains very low during early embryonic development, while that in the whole embryo increases rapidly. This is indicative of the alkali-insoluble material being deposited and accumulating in the cell wall. In preparing pure fractions of cell walls and cytoplasm, only 50-60% of the total alkali-insoluble material was recovered. This was because of losses occurring in the cell wall fraction when walls were repeatedly washed, homogenized, and the washes strained through Miracloth to remove cell wall fragments. Hence, quantitative

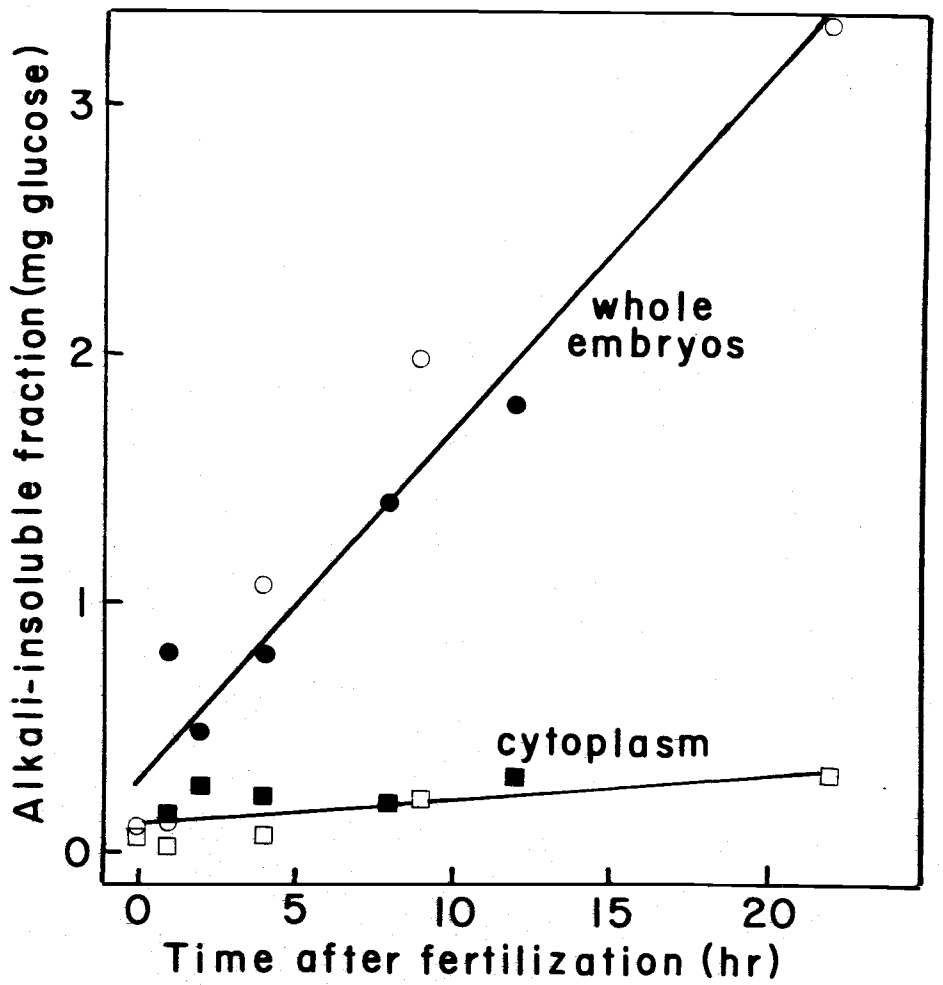



Figure 6. Amount of alkali-insoluble fraction, measured in mg of glucose, in whole embryos (O—O) and cytoplasm (□—□) of F. distichus at various times after fertilization. The figure combines the data of two separate experiments.

recovery of cell walls was not possible and a direct determination of the alkali-insoluble material in this fraction was not accomplished. However, since stringent precautions were taken to exclude any contaminating walls or wall fragments from the cytoplasmic fraction (straining the washings repeatedly through Miracloth as well as microscopic examination), data obtained for the quantity of alkali-insoluble material in the cytoplasmic fraction are reliable. Therefore, the amount of alkali-insoluble material in the cell wall fraction represents the difference between that in the whole embryo and that in the cytoplasmic fraction.

From these data it appears that the increase in alkali-insoluble material occurs in the cell wall fraction and synthesis and/or assembly may occur extracellularly. Although the trace amount of alkali-insoluble material in the cytoplasm is likely to be caused by very small fragments of cell walls, not detectable by microscopic examination, one cannot exclude the possibility that a small amount is synthesized in the cytoplasm, rapidly transported to the plasma membrane, and accumulated extracellularly into the wall.

## B. Composition of Alkali-Insoluble Material

What is the nature of the insoluble material remaining after treatment of cell walls with hot alkali? To answer this question two approaches were utilized:

(i) To chemically hydrolyze the material using standard procedures for proteins and polysaccharides and identify the monomers released by paper, thin-layer and gas chromatography.

(ii) To utilize highly purified hydrolases to characterize the nature of the linkages in the polymer by identifying the hydrolytic products released. The basic procedure was to isolate alkali-insoluble material from 24 or 48 hour embryos for analyses. $^{14}C$-labeled alkali-insoluble material was used as substrate to increase the sensitivity to detect hydrolytic products released by chemical hydrolysis and purified enzymes.

1. Acid Hydrolysis

a. Protein. No ninhydrin-reacting material was detected in the hydrolysate (6 N HCl) of 500 μg of alkali-insoluble material, indicating the absence of protein in this cell wall fraction. Negative results were also obtained when the unhydrolyzed and HCl-hydrolyzed material was tested by the Lowry method for protein determination.

b. Polysaccharides. Hydrolysis by sulfuric acid indicates that the major monomer unit of the alkali-insoluble polysaccharide is glucose. The hydrolysate of alkali-insoluble material extracted from $^{14}C$-labeled embryos co-chromatographs with glucose on thin-layer

plates in solvent systems (1) and (2), as well as on paper using solvent system (3). In solvent system (1), 95% of the counts in the hydrolysate migrated the same distance as glucose. A faint spot migrating the same distance as a glucose standard is the only sugar that can be detected with anisaldehyde spray reagent. When chromatograms were sprayed with naphthoresorcinol reagent, no spots developed indicating the absence of uronic acids (Stahl, 1969). When the acid hydrolysate from alkali-insoluble material was subjected to gas chromatography, 84% of the hydrolytic products co-chromatographed with α- and β-glucose standards and the remaining 16% was found in fucose and xylose (Figure 7).

2. Enzyme Hydrolysis

$^{14}C$-labeled alkali-insoluble material was subjected to enzymatic hydrolysis using purified endo-β-1,3 and endo-β-1,4-glucanases. Release of cellobiose (2), a β-1,4-disaccharide of glucose, using an endo-β-1,4-glucanase would be indicative of the presence of β-1,4-linkages (1), whereas, release of laminaribiose (4), a β-1,3-disaccharide of glucose, using an endo-β-1,3-glucanase would be indicative of the presence of β-1,3-linkages (3) in the alkali-insoluble material.

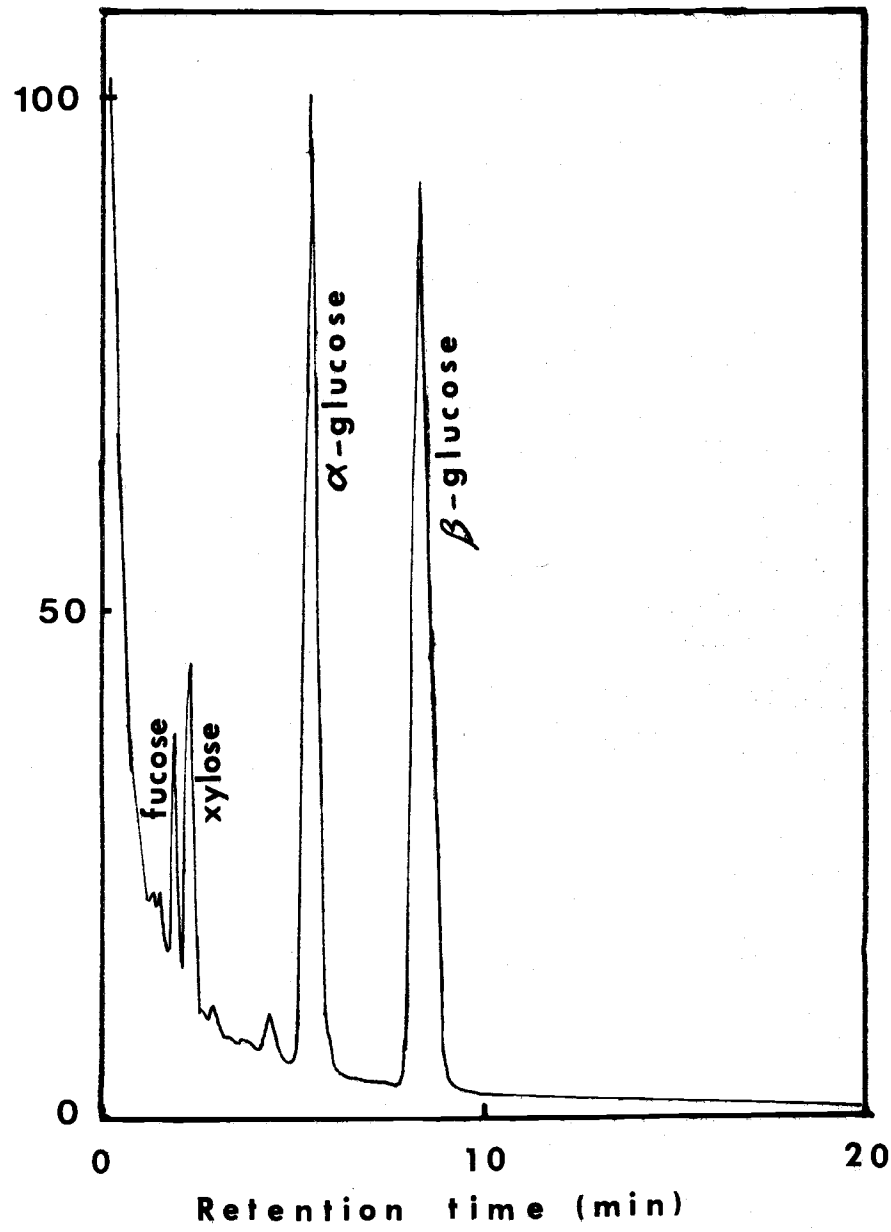
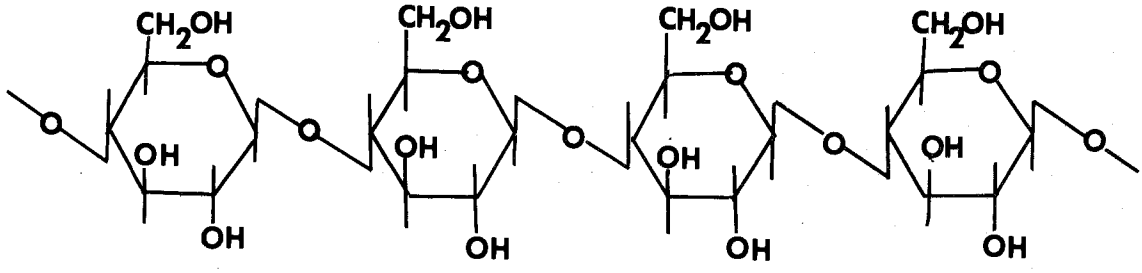
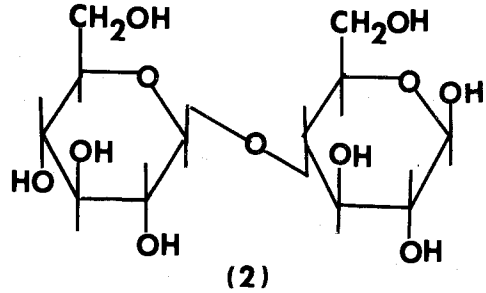



Figure 7. Gas chromatographic scan of the silyl derivatives from the acid hydrolysate of alkali-insoluble material. Glucose is the major hydrolytic product from F. distichus embryos with the remainder in two minor peaks corresponding to fucose and xylose.

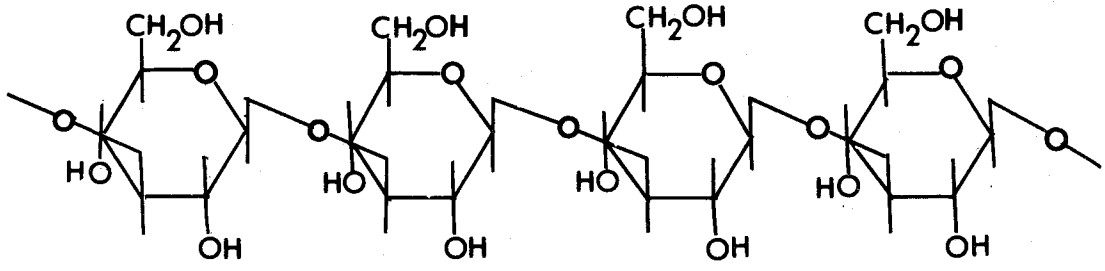


(1)

ENDO–β 1,4 GLUCANASE

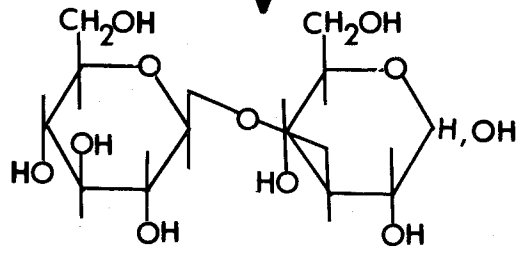


(2)



(3)

ENDO–β 1,3 GLUCANASE



(4)

The products of enzymatic hydrolysis were separated by descending paper chromatography in solvent system (3) and their migration distances compared with laminaribiose, cellobiose and glucose. As seen in Figure 8, 86% of the radioactivity that migrated after incubation of $^{14}C$ alkali-insoluble material with endo-β-1,4-glucanase appeared in a peak corresponding to cellobiose, and no radioactivity was detected in the area of laminaribiose when the material was treated with an endo-β-1,3-glucanase. Radioactivity remaining at the origin in the endo-β-1,4-glucanase treated sample probably represents small polymers solubilized by the endoenzyme during the incubation period and not removed by centrifugation of the reaction mixtures. When control incubation mixtures containing only $^{14}C$-labeled alkali-insoluble material in buffer were chromatographed, no radioactivity was detected in the regions corresponding to cellobiose, laminaribiose or glucose. These results strongly suggest that the alkali-insoluble material contains a β-1,4-linked glucan polymer with no detectable β-1,3-linkages under the experimental conditions employed. Therefore, the alkali-insoluble material possesses the solubility characteristics and linkages of cellulose.

## C. Cellulose Synthesis and Deposition

Alkali-insoluble polysaccharide (i.e., cellulose) appears to be deposited into the newly forming cell walls of zygotes very rapidly

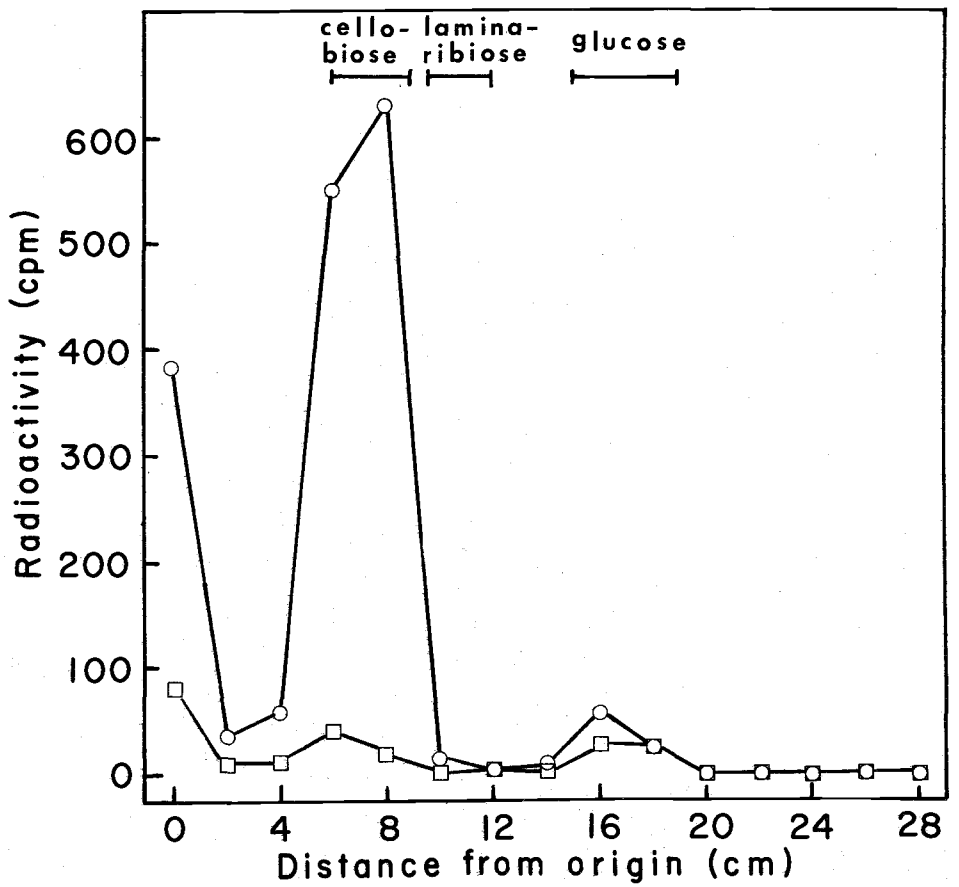



Figure 8. Distribution of radioactivity on a paper chromatograph from the enzyme-hydrolyzed $^{14}C$-alkali-insoluble material from 24 hour F. distichus embryos. Cellobiose was the major disaccharide released when alkali-insoluble polysaccharide was incubated with an endo-β-1,4-glucanase (O—O), while no detectable laminaribiose was observed with an endo-β-1,3-glucanase (□—□).

after fertilization as evidenced by the presence of this component in isolated cell wall material within 60 minutes following fertilization. To elucidate the levels of control operative during cellulose deposition into the new cell wall, one must determine if cellulose is synthesized de novo, or if its synthesis represents the polymerization of preformed oligosaccharide units present in unfertilized eggs. Also, if cellulose is newly synthesized, are the enzymes responsible for its synthesis preformed in the egg or newly synthesized during the large increase of protein synthesis after fertilization (Peterson and Torrey, 1968)? Zygotes incubated in radioactively labeled precursors such as $NaH^{14}CO_3$ or $^3$H-glucose should incorporate $^{14}$C and $^3$H into cellulose if newly synthesized polymer is formed, and, if this incorporation is dependent upon formation of new enzymes responsible for cellulose synthesis, inhibitors of protein synthesis such as cycloheximide, should prevent the post-fertilization increase in cellulose accumulation.

Incubation of zygotes in $NaH^{14}CO_3$ continuously during the early stages of development results in accumulation of $^{14}$C into cellulose. The data in Table 1 demonstrates that the majority of cellulose present by 24 hours is synthesized and deposited within the first 4-5 hours after fertilization and that a constant proportion of the total cellulose is labeled. Zygotes incubated in $^3$H-D-glucose also accumulate radioactivity in cellulose (Table 2).

Table 1. Accumulation of $^{14}C$ from $NaH^{14}CO_3$ (1 μCi/ml) into the ethanol-soluble and cellulose fraction of F. distichus embryos harvested at different times after fertilization.

| Time of Label[1] | Radioactivity | | Amount of Cellulose | Specific Activity |
|---|---|---|---|---|
| | Ethanol-Soluble | Cellulose | | |
| (hrs. after fertilization) | (cpm) | (cpm) | (μg glucose) | (cpm/μg glucose) |
| 0-3 | 84,108 | 10,376 | 140 | 74.1 |
| 0-4.5 | 105,592 | 16,054 | 190 | 84.5 |
| 0-24 | 112,276 | 21,765 | 280 | 77.7 |
| 0-48 | 82,588 | 22,927 | 440 | 52.1 |

[1]Each sample contained equal numbers of embryos.

Table 2. Accumulation of $^{3}H$ from $^{3}H$-D-glucose (2 μCi/ml) into the ethanol-soluble and cellulose fractions of F. distichus embryos harvested at different times after fertilization.

| Time of Label[1] | Radioactivity | | Amount of Cellulose | Specific Activity |
|---|---|---|---|---|
| | Ethanol-Soluble | Cellulose | | |
| (hrs. after fertilization) | (cpm) | (cpm) | (μg glucose) | (cpm/μg glucose) |
| 0-3 | 11,280 | 510 | 75 | 6.8 |
| 0-10 | 68,010 | 2,055 | 330 | 6.2 |
| 0-24 | 80,640 | 3,640 | 775 | 4.7 |

[1]Each sample contained equal numbers of embryos.

To determine the rate of synthesis at different times after fertilization, zygotes of F. distichus were given 60 minute pluses of $NaH^{14}CO_3$ (1 μCi/ml). The rate of incorporation into cellulose is highest during the first hour following fertilization, decreases over the next five hours, and remains at a low but constant rate during rhizoid formation and cell division (Figure 9).

To study the kinetics of cellulose synthesis during the first three hours after fertilization, the dioecious species F. vesiculosus, was used so that the time of fertilization could be more precisely determined. Zygotes of F. vesiculosus were given 20 minute pulses of $NaH^{14}CO_3$ (0.75 μCi/ml) at different times during the first three hours. Incorporation of $^{14}C$ into cellulose begins almost immediately after fertilization with the highest rate during the first 20 minute pulse interval (Figure 10). The rate decreases markedly after the first hour. No detectable cellulose was found in unfertilized eggs and sperm pulsed separately for 20 minutes with $NaH^{14}CO_3$.

Cycloheximide, at concentrations shown by Quatrano (1968b) to reversibly inhibit protein synthesis, did not significantly reduce the amount of cellulose in zygotes treated between 0 and 14 hours. In addition, incorporation of $^{14}C$ into cellulose was not significantly inhibited by cycloheximide during the first 10 hours after fertilization (Tables 3 and 4). Zygotes of F. vesiculosus were used in this experiment and eggs and sperm were pre-treated with cycloheximide for

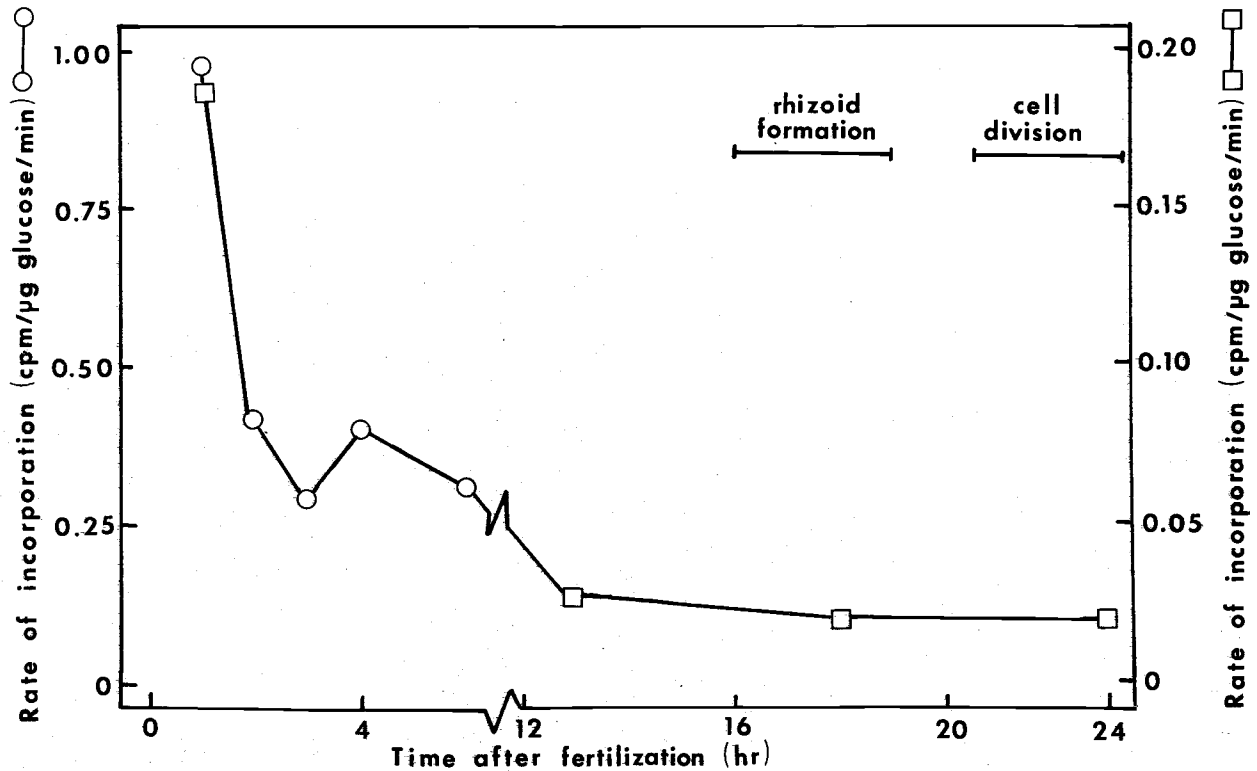



Figure 9. Rate of incorporation of $^{14}C$ from $NaH^{14}CO_3$ (1 µCi/ml) into the cellulose fraction of F. distichus embryos at different times after fertilization. A 60 minute pulse was given at the indicated times. The figure combines the data of two separate experiments, comparing the rate of incorporation at early times after fertilization (O—O) and at times up to 23 hours after fertilization (□—□). Rhizoid formation began at approximately 17 hours and cell division at 22 hours in these experiments.

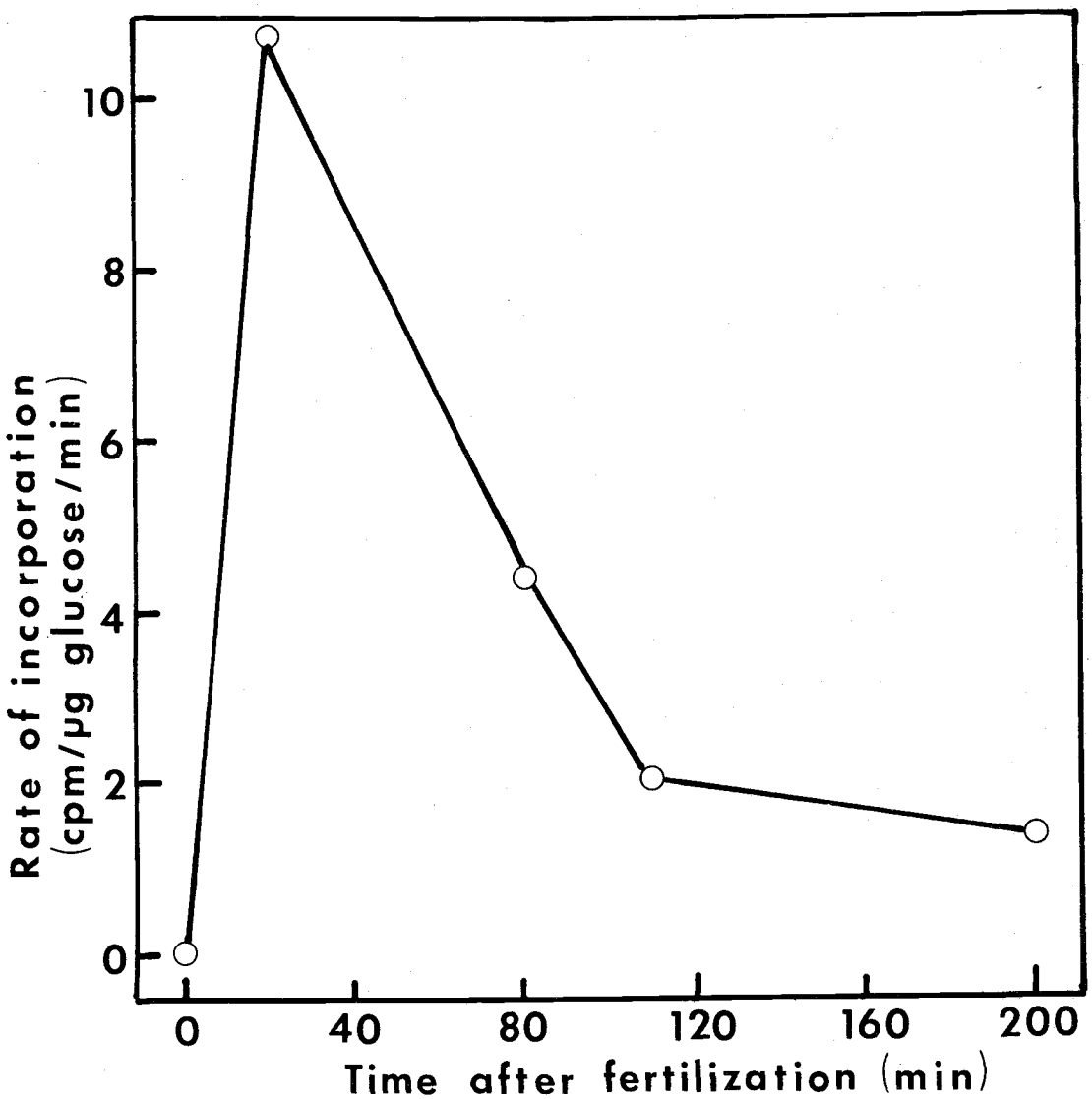



Figure 10. Rate of incorporation of $^{14}C$ from $NaH^{14}CO_3$ (0.75 µCi/ml) into the cellulose fraction of F. vesiculosus embryos at different times after fertilization. A 20 minute pulse was given at the indicated times. The data point at 0 minutes represents both unfertilized eggs and sperm pulsed for 20 minutes with $NaH^{14}CO_3$ and these contained no detectable alkali-insoluble material.

Table 3. Effect of cycloheximide (2-5 μg/ml) on the amount of cellulose deposited in cell walls of F. vesiculosus and F. distichus embryos.

| Treatment Interval[1] | Amount of Cellulose | | Inhibition |
|---|---|---|---|
| | Control | Treated | |
| (hrs after fertilization) | (μg glucose) | (μg glucose) | (%) |
| F. vesiculosus | | | |
| 0-6 | 105 | 95 | 10 |
| 0-14 | 150 | 125 | 20 |
| F. distichus | | | |
| 1-4 | 160 | 140 | 14 |
| 4-9 | 160 | 130 | 23 |
| 9-14 | 200 | 210 | -5 |

[1]Each sample contained equal numbers of zygotes.

Table 4. Effect of cycloheximide (1 μg/ml) on the incorporation of $^{14}C$ from $NaH^{14}CO_3$ (1 μCi/ml) into the cellulose fraction of F. vesiculosus embryos.

| Treatment Interval | Radioactivity in Cellulose | | Inhibition |
|---|---|---|---|
| | Control | Treated | |
| (hrs after fertilization) | (cpm) | (cpm) | (%) |
| 0-0.5 | 7,056 | 6,974 | 1.2 |
| 0-1.5 | 19,549 | 17,878 | 9.9 |
| 0-10 | 61,591 | 64,923 | -5.1 |

30 minutes prior to the time of eggs and sperm mixing. This was done to insure that the inhibitor would be present in cells during fertilization.

Various concentrations of a number of specific metabolic inhibitors were without effect on the synthesis and deposition of cellulose into the cell wall (Table 5). However, carbonylcyanide p-trifluoromethoxy phenylhydrazone (FCCP), an uncoupler of oxidative phosphorylation (Cramer et al, 1971) at $10^{-6}$ M, prevented cell wall deposition and reduced cellulose synthesis by 67%.

In summary, at least a constant proportion of the cellulose which is deposited into the cell wall is newly synthesized and the enzymes involved in this synthesis appear to be present in the egg at the time of fertilization. New synthesis of these enzymes is not required for the energy-requiring process of cellulose deposition into the new cell wall of the zygote.

## D. Substrates for Cellulose Synthesis

As shown in the previous section, cellulose synthesis and deposition into the cell wall of Fucus zygotes occurs at a rapid rate shortly after fertilization. These events take place in an inorganic medium, containing only KCl, $CaCl_2$, $Na_2SO_4$ and $MgCl_2$ (Quatrano, 1974). No external carbon source is required, nor is light needed for wall development. However, the process of cell wall formation

Table 5. Inhibitors without effect on cellulose deposition or synthesis.

| Inhibitor | Concentration Range Tested | Site of Inhibition | Reference |
|---|---|---|---|
| Cycloheximide | 0.1 to 100 μg/ml | Protein synthesis | Quatrano, 1968b |
| Colchicine | 0.5 to 10 mg/ml | Microtubule assembly | Quatrano, 1973 |
| Cytochalasin B | 0.5 to 100 μg/ml | Microfilament assembly (?) | Quatrano, 1973 |
| Deoxyglucose | 0.5 to 100 mM | Glucan synthesis | Zonneveld, 1973 |
| Coumarin | $10^{-5}$ to $10^{-3}$ M | Cellulose synthesis | Hara et al, 1973 |
| Dichlorobenzylnitrile | $10^{-5}$ to $10^{-3}$ M | Cellulose synthesis | Hogetsu et al, 1974 |

requires an endogenous energy source, as demonstrated by the inhibition of cellulose synthesis by FCCP, an uncoupler of phosphorylation (see Section IIIC). Also, zygotes are capable of using a carbon source for cellulose synthesis as demonstrated by the incorporation of label from exogenously supplied $NaH^{14}CO_3$ or $^3H$-glucose into cellulose (see Section IIIC). The endogenous carbon units and energy required for cellulose synthesis may be supplied by dark $CO_2$-fixation which has been reported in brown algae (Nisizawa et al, 1971), breakdown of a storage polysaccharide, or by a combination of these metabolic processes.

The water-soluble β-1,3-glucan, laminarin, is found abundantly in species of the Phaeophyta as a food reserve material (Lewin, 1962). Percival and McDowell (1967) report that laminarin constitutes approximately 7% of the dry weight of Fucus fronds. If present in eggs and young embryos, laminarin could provide a source of energy and carbon substrates for cell wall synthesis.

Thus, it was of interest to determine (i) if laminarin is present in unfertilized eggs; (ii) if so, whether a decrease in total cellular laminarin could be detected during the period when the cell wall is synthesized; and (iii) if zygotes contain an enzyme that is capable of hydrolyzing laminarin.

Laminarin can be extracted from embryos by the procedure of Mian and Percival (1973), who found that the soluble glucans of brown

seaweeds are contained in the aqueous calcium chloride extract. The uncharged laminarin can be purified from the charged, sulfated fucans in this fraction by elution with water from a DE-52-cellulose column. When a fraction was obtained from Fucus embryos by this procedure and acid hydrolyzed, only glucose was detected on thin-layer plates in solvent system (1). No fucose was detected in the same hydrolysate. The laminarin extract was also subjected to enzymatic hydrolysis with purified endo-β-1,3-glucanase. When separated by descending paper chromatography in solvent system (4), the major hydrolysis product co-chromatographed with a laminaribiose standard and a minor product migrated the same distance as a glucose standard.

If the products of laminarin breakdown are used as substrates for the rapid cell wall synthesis following fertilization, a decrease in total cellular laminarin 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 seven hours in unlabeled sea water. The laminarin and cellulose fractions of these zygotes were extracted and compared to that from an equal volume of pre-labeled, unfertilized eggs. As shown in Table 6, both the total amount of laminarin, as measured by glucose content, and the radioactivity in this fraction decreases during the early period following fertilization, while the total amount of cellulose as well as the cpm in cellulose increases. Since laminarin breakdown appears to occur

concomitantly with cell wall formation, one possible use for the products of laminarin hydrolysis could be synthesis of new cell wall polysaccharides, such as cellulose. Because of the non-specificity of the radioactive label used in this experiment, it is not possible to determine whether the $^{14}C$ that accumulates in newly synthesized cellulose represents glucan units from laminarin hydrolysis, soluble sugar pools, hydrolysis of other storage polysaccharides or dark $CO_2$-fixation. Most likely a combination of these processes provide the substrates for cellulose synthesis.

Table 6. Decrease of laminarin and increase of cellulose in F. vesiculosus zygotes between 0 and 7 hours after fertilization. Eggs were labeled with $^{14}C$ from $NaH^{14}CO_3$ (2 μCi/ml) during oogenesis and incubated in unlabeled sea water following fertilization.

| Time of Incubation | Radioactivity in Laminarin | Amount of Laminarin | Radioactivity in Cellulose | Amount of Cellulose |
|---|---|---|---|---|
| (hrs after fertilization) | (cpm) | (mg glucose) | (cpm) | (mg glucose) |
| 0 | 100,080 | 42.5 | 6,560 | 0.13 |
| 7 | 40,810 | 11.9 | 28,840 | 0.73 |

Homogenates of Fucus embryos possess β-glucosidase activity as measured by release of p-nitrophenyl from the synthetic substrate p-nitrophenyl-β-D-glucopyranoside (Quatrano, 1974). Approximately 80-90% of the β-glucosidase activity is found in a particulate fraction which can be solubilized with 0.2% Triton-X. Hydrolysis of a storage

glucan in eggs by this enzyme could provide important products, e.g., glucose, which could be used as a carbon source as well as a substrate for ATP formation to synthesize cell wall material. Thus, it was of interest to determine if this enzyme preparation was capable of hydrolyzing a naturally occurring β-glucan storage polymer found in eggs, namely laminarin.

When a Triton-X-solubilized enzyme preparation was incubated with laminarin, 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-laminarin was used as substrate. The soluble enzyme preparation released increasing quantities of glucose from $^{14}$C-laminarin, extracted from Fucus zygotes, over a 24-hour period (Table 7). Glucose was the only hydrolysis product detected in reaction mixtures which were subjected to paper chromatography. These results provide evidence that the enzyme is an exo-β-1,3-glucanase.

Table 7. Release of glucose from $^{14}$C-laminarin by a crude enzyme preparation from Fucus zygotes. Reaction products were determined by paper chromatography of the incubation mixtures.

| Time of Incubation | Glucose Released[1] |
|---|---|
| (hrs) | (cpm) |
| 0 | 43 |
| 4 | 204 |
| 16 | 676 |
| 24 | 792[2] |
| -enzyme | 0 |

[1] All counts which migrated co-chromatographed with a glucose standard.

[2] This represents 17% of the total cpm in the reaction mixture.

# IV. DISCUSSION

A method has been demonstrated for isolating intact cell walls free of cytoplasmic contamination from Fucus zygotes. Although the isolation procedure involves mechanical forces and aqueous washes to remove cytoplasmic contents, the walls remain intact and retain the morphological characteristics of particular developmental stages. These characteristics are retained even after treatment of the isolated walls with hot alkali. Observations such as this suggest that the wall component resistant to alkali treatment is responsible for a particular cell form and that the hot alkali-soluble matrix polysaccharides, such as alginic acid and fucoidan, are not directly involved in the maintenance of cellular shape. Although purified cell walls have been isolated from a number of organisms (see Section IB), this study represents the first case of normal cell wall changes in shape to be retained in purified walls and correlated with a particular polysaccharide fraction. The techniques and data obtained can now be used for biochemical and biophysical approaches to understand the assembly and subsequent alteration of this complex network of polysaccharides in relation to a specific shape change.

Solubility properties, sugar composition and the type of glycosidic linkage identified the alkali-insoluble component of the cell walls as cellulose. Thin layer chromatography of the acid hydrolysate

of alkali-insoluble material revealed only glucose. Gas chromatography of the same acid hydrolysate revealed primarily glucose (84%) with the remaining sugars identified as xylose and fucose. Enzymatic hydrolysis of the polymer with endo-β-1,4 and endo-β-1,3-glucanases released only cellobiose, indicating that the material contains β-1,4-linked glucose units and no detectable β-1,3-linkages. Although other linkages may be present in this material, neither α-glucans nor β-glucans, other than laminarin (β-1,3) and cellulose (β-1,4) have been reported in the Phaeophyta (Percival and McDowell, 1967).

Although cellulose is deposited into the cell wall immediately after fertilization, the ordered structure of this polymer into microfibrils and its incorporation into a stable wall complex with other polysaccharides is apparently not complete until four hours after fertilization. Most of the cell walls of embryos collected 0-4 hours after fertilization fragment during the purification procedure whereas those collected at later stages of development tend to remain intact. Quatrano (1968a) has observed that the cell walls of F. vesiculosus develop the property of birefringence, which is indicative of an ordered, crystalline structure, during the first four hours after fertilization. In populations of zygotes one-hour old, only a small percentage of cells appear birefringent, but 4 hours after fertilization, 100% of the cells are birefringent.

Although the matrix polysaccharides appear not to be necessary for maintenance of cell shape, it is possible that they contribute to the initial assembly of the wall, or that a unique non-cellulosic polymer is involved with orienting cellulose microfibrils into a stable shape. In higher plants, a xyloglucan has been identified which is covalently linked to pectic polysaccharides and hydrogen-bonded to cellulose (Bauer et al, 1973; Valent and Albersheim, 1974). Cellulose in Fucus walls may likewise be bound to a similar polymer, for example, a xylofucan described by Mian and Percival (1973). This molecule could be a type of fucan deposited during the first four hours that can serve to link cellulose chains into a stable form. Ley and Quatrano (1973) determined the carbohydrate composition of purified cell walls of F. vesiculosus embryos at different times after fertilization and found that the level of fucans in the wall increased linearly from 0-4 hours, paralleling the appearance of birefringence in the wall. These fucans alone are not birefringent (Percival and McDowell, 1967). A polymer of this type covalently bound to cellulose may also account for the significant amount of xylose and fucose residues detected by gas chromatography in cellulose hydrolysates from zygote walls. Hence, the three dimensional associations of cellulose molecules with a matrix polysaccharide during the first four hours may account for its ability to maintain the shape of the cell after treatment with hot alkali.

No protein was detected in the cellulose fraction from the isolated walls of Fucus, although glycoproteins appear to be responsible, at least in part, for the structural framework of cell walls of some algae. The cell wall of Chlamydomonas contains glycoproteins assembled into a geometrical lattice which has been described as an 80° parallelogram (Hills et al, 1973). Extracellular scales of the chrysophycean alga Pleurochrysis consist of cellulose covalently linked to peptide moieties. The protein content of the alkali-purified scale material is 32% per dry weight (Herth et al, 1972).

Cellulose deposition into the newly forming cell wall of Fucus occurs within 20 minutes after fertilization. Based on solubility properties, cellulose is not detectable in eggs but increases in amount during the first four hours after fertilization. Incorporation of radioactive precursors indicated that at least a part of this cellulose is newly synthesized after fertilization. Most, if not all, of this cellulose is found associated with the cell wall and not cytoplasm. Although traces of cellulose found in the cytoplasm are thought to be due to contamination from small cell wall fragments, one cannot exclude the possibility that very small amounts of cellulose are synthesized in the cytoplasm and rapidly transported to the plasma membrane for deposition and accumulation extracellularly. Although the site of cellulose synthesis in Fucus is obscure, the Golgi appears to be the site of synthesis of a cellulosic glycoprotein in Pleurochrysis (Brown, 1969;

Herth et al, 1972) and of cellulose in germinating Petunia pollen (Engels, 1974). Chitin microfibril synthesis in Mucor appears to initiate at the site of a membrane but may continue extracellularly (Ruiz-Herrera and Bartnicki-Garcia, 1974). Vesicles derived from Golgi have been observed to fuse with the plasmalemma and deposit undefined fibrillar material extracellularly in many plants (c.f., Sassen, 1964; Van der Woude et al, 1971) including Fucus (Jaffe, 1968; Quatrano, 1968a). All of these findings are consistent with a working hypothesis that cellulose chains are synthesized in the Golgi or Golgi vesicles and assembled into microfibrils extracellularly.

Attention has been given to the possible role of microtubules in the control of cell wall deposition (c.f. Hepler and Palevitz, 1974). Removal of microtubules by colchicine causes aberrant wall deposition and, specifically, disruption of the cellulose microfibril pattern, during differentiation of xylem cells (Pickett-Heaps, 1967; Hepler and Fosket, 1971). Colchicine does not affect the quantity of cellulose deposited into the cell wall of Fucus embryos even though the drug does prevent cell division in this organism (Quatrano, 1973). This finding confirms the hypothesis of Hepler and Palevitz (1974) that although microtubules appear to play a role in microfibril orientation, they do not affect cellulose synthesis since they are notably absent from certain cells where active wall synthesis and deposition occur, such as root hairs, pollen tubes and fungal hyphae.

Coumarin and dichlorobenzylnitrile, which have been shown to inhibit auxin-induced cellulose synthesis in higher plants (Hara et al, 1973; Hogetsu et al, 1974), did not affect cellulose synthesis in Fucus zygotes. However, since auxins are involved in cell wall expansion in these systems, these inhibitors may be affecting hormonal synthesis, or transport, and only cellulose synthesis secondarily. No auxin requirement has yet been demonstrated for rhizoid elongation in Fucus. An analog of glucose, 2-deoxyglucose, inhibited cell wall regeneration in yeast protoplasts (Farkas et al, 1970) and prevented cleistothecium formation in Aspergillus (Zonneveld, 1973) but had no effect on cellulose synthesis or deposition in Fucus zygotes.

One objective of the present study was to obtain information of the genetic regulation of cellulose synthesis and assembly into the new cell wall. The highest rate of cellulose synthesis appears to occur in the first 20 minutes following fertilization of the Fucus egg, although synthesis and deposition of cellulose into the cell wall continues throughout subsequent development. The rapid initial rate suggests that the genetic information for cellulose synthesis and assembly into the wall is already present in the egg at the time of fertilization. Cycloheximide, at a concentration which inhibits protein synthesis in the cell by 95% (Quatrano, 1968b), had no effect on the rate of synthesis or quantity of cellulose deposited into the wall. Cycloheximide was also found to have no effect on fibril formation in

the regenerating walls of yeast protoplasts but it did inhibit synthesis of wall matrix (Farkas et al, 1970). Actinomycin D, at a concentration which inhibits RNA synthesis by 75%, has no effect on the ability of cell walls to become birefringent in F. vesiculosus zygotes (Quatrano, 1968a). These findings demonstrate that although these inhibitors actively block cellular RNA and protein synthesis, they do not affect cellulose synthesis or deposition into the new cell wall of zygotes. Thus, since new RNA and protein synthesis are not required for formation of a cellulosic wall, the process of cellulose synthesis and deposition is not controlled at the genetic level (i.e., transcription and translation).

The fact that cellulose-synthesizing enzyme activity changes independently of genetic regulation is an unusual case in embryogenesis. Various enzyme activities that change during sea urchin embryogenesis, including ribonucleotide reductase activity (Noronha et al, 1972), and enzymes controlling synthesis of tubulin (Raff et al, 1972) and histones (Kedes et al, 1969) are regulated at the level of transcription or translation. The only other case known in which a change in enzyme activity during embryogenesis is not regulated genetically is aspartate aminotransferase in early sea urchin development (Neyfakh, 1971).

Cellulose-synthesizing enzymes could be activated by intracellular ionic changes that occur in the fucoid egg following

fertilization. Allen et al (1972) have shown that intracellular $K^+$ increases substantially during the first three hours after fertilization in zygotes of Pelvetia, a species closely related to Fucus which undergoes similar embryogenesis. Intracellular $Cl^-$ also increases significantly following fertilization. If the enzymes concerned with cellulose synthesis are located at the plasma membrane (c.f., Northcote, 1969a; Ruiz-Herrera and Bartnicki-Garcia, 1974), changes in membrane potential at the time of fertilization of Pelvetia eggs (Weisenseel and Jaffe, 1972), may play a direct role in enzyme activation or cause changes in intracellular levels of certain ions, which would then activate synthetase enzymes. Another possibility may be that energy is limiting and the increased respiration rate shown to occur after fertilization in Fucus (Whittaker, 1931) provides high-energy nucleotides for the initial steps of cellulose synthesis. Whatever the mechanism of activation or the site of cellulose synthesis in Fucus, isolation of a cell-free enzyme system for cellulose synthesis is the first step to approach these questions and further our understanding of the processes involved in assembly of the shape-stabilizing component of the cell wall.

FCCP, an uncoupler of oxidative phosphorylation, prevents cellulose synthesis and wall deposition in Fucus zygotes. This is expected if cellulose assembly into the cell wall depends on synthesis via the conventional pathway involving a sugar nucleoside precursor

(Barber et al, 1964). However, the energy necessary for cellulosic wall formation in Fucus zygotes may not be required exclusively for synthesis. If the cell wall is deposited by the crystallization mechanism proposed by Nečas (1971) for yeast wall regeneration, or by a self-assembly mechanism as proposed for the glycoprotein lattice of Chlamydomonas cell walls (Hills, 1973), energy may be required to transport glucose or oligosaccharides across the plasma membrane. Crystallization or self-assembly of these molecules, which could take place extracellularly, may also require energy.

The β-1,3-glucan, laminarin, may be a source of energy and carbon substrates for cellulose synthesis. Total cellular laminarin decreases during early embryonic development while cellulose is being deposited into the newly forming cell wall. Zygotes possess a glucanase which is capable of hydrolyzing native laminarin to glucose. The enzyme is not active on native cellulose or the synthetic β-1,4-glucan polymer, carboxymethylcellulose. Thus, zygotes possess a mechanism for laminarin breakdown, which in turn could provide glucose for cellulose synthesis and for ATP formation. Whittaker (1931) noted a large increase in respiration after fertilization of Fucus eggs. Approximately 50 times more glucose is released from laminarin than can be accounted for in newly synthesized cellulose during the first seven hours after fertilization (see Table 6). Therefore, it is possible that the products of laminarin breakdown are used

for ATP production. Evidence for the ability of brown algae to fix $CO_2$ into polymers in the dark (Nisizawa et al, 1971) suggests that a similar mechanism may be operating in Fucus zygotes to provide carbon substrates for cellulose synthesis.

In summarizing the results of the present study, the following picture of cell wall formation in Fucus zygotes emerges: The component of the cell wall responsible for maintenance of cell shape is cellulose, and its deposition into the cell wall begins almost immediately after fertilization. This requires enzyme activity and cellular energy for cellulose synthesis and wall formation, since no cellulose or wall is found in the egg cell. The enzyme is not regulated by controls at transcription or translation, but at some other level, probably enzyme activation. Laminarin breakdown by β-glucanase may provide substrates used for cellulose synthesis at the time of cell wall formation.

# BIBLIOGRAPHY

Allen, R.D., L. Jacobsen, J. Joaquin and L.F. Jaffe. 1972. Ionic concentrations in developing Pelvetia eggs. Devel. Biol. 27:538-545.

Balinsky, B.I. 1970. "An Introduction to Embryology." W.B. Saunders Co., Philadelphia.

Barber, G.A., A.D. Elbein and W.Z. Hassid. 1964. The synthesis of cellulose by enzyme systems from higher plants. J. Biol. Chem. 239:4056-4061.

Bauer, W.D., K.W. Talmadge, K. Keegstra and P. Albersheim. 1973. The structure of plant cell walls. II. The hemicellulose of the walls of suspension-cultured sycamore cells. Plant Physiol. 51:174-187.

Blackburn, J. 1968. "Amino acid determination." Marcel Dekker, New York.

Brawley, S.H., R. Wetherbee and R.S. Quatrano. 1974. Cell division and rhizoid formation during embryogenesis in Fucus vesiculosus (Phaeophyta). J. Phycol. 10 (suppl):8.

Brown, R.M. 1969. Observations on the relationship of the Golgi apparatus to wall formation in the marine alga, Pleurochrysis scherffelii Pringsheim. J. Cell Biol. 41:109-123.

Brown, R.M., W.W. Franke, H. Kleinig, H. Falk and Peter Sitte. 1969. Cellulosic wall component produced by the Golgi apparatus of Pleurochrysis scherffelii. Science 166:894-896.

Burgess, J. and E. N. Fleming. 1974. Ultrastructural observations of cell wall regeneration around isolated tobacco protoplasts. J. Cell Sci. 14:439-449.

Clark, A.F. and C.L. Villemez. 1972. The formation of β, 1, 4 glucan from UDP-α-D-glucose catalyzed by a Phaseolus aureus enzyme. Plant Physiol. 50:371-374.

Cocking, E.C. 1972. Virus uptake, cell wall regeneration and virus multiplication in isolated plant protoplasts. Ann. Rev. Plant Physiol. 23:29-50.

Cocucci, A. and W.A. Jensen. 1969. Orchid embryology: Megagametophyte of Epidendrum scutella following fertilization. Amer. J. Bot. 56:629-640.

Cramer, W.A., H.N. Fan and H. Bohme. 1971. High and low potential states of the chloroplast cytochrome b-559 and thermodynamic control of non-cyclic electron transport. Bioenergetics 2:289-303.

Delmer, D.P., C.A. Beasley and L. Ordin. 1974. Utilization of nucleoside diphosphate glucoses in developing cotton fibers. Plant Physiol. 53:149-153.

Diboll, A.G. 1968. Fine structural development of the megagametophyte in Zea mays following fertilization. Amer. J. Bot. 55:797-806.

Engels, F.M. 1974. Relation to cell wall synthesis in germinating Petunia pollen. IV. Identification of cellulose in pollen tube walls and Golgi vesicles by x-ray diffraction. Acta Bot. Neerl. 23:209-215.

Farkas, V., A. Svoboda and S. Bauer. 1970. Secretion of cell wall glycoproteins by yeast protoplasts. The effect of 2-deoxy-D-glucose and cycloheximide. Biochem. J. 118:755-758.

Hanke, D.E. and D.H. Northcote. 1974. Cell wall formation by soybean callus protoplasts. J. Cell Sci. 14:29-50.

Hara, M., N. Umetsu, C. Miyamoto and K. Tamari. 1973. Inhibition of the biosynthesis of plant cell wall materials, especially cellulose biosynthesis, by coumarin. Plant and Cell Physiol. 14:11-28.

Hassid, W.Z. 1969. Biosynthesis of oligosaccharides and polysaccharides in plants. Science 165:137-144.

Hepler, P.K. and D.E. Fosket. 1971. The role of microtubules in vessel member differentiation in Coleus. Protoplasma 72:213-236.

Hepler, P.K. and B.A. Palevitz. 1974. Microtubules and microfilaments. Ann. Rev. Plant Physiol. 25:309-362.

Herth, W., W.W. Franke, J. Stadler, H. Bittiger, G. Keilich and R.M. Brown. 1972. Further characterization of the alkali-stable material from the scales of Pleurochrysis scherffelii: A cellulosic glycoprotein. Planta 105:79-92.

Hills, G.J. 1973. Cell wall assembly in vitro from Chlamydomonas reinhardi. Planta 115:17-23.

Hogetsu, T., H. Shibaoka and M. Shimokoriyama. 1974. Involvement of cellulose synthesis in actions of gibberellin and kinetin on cell expansion. 2,6-Dichlorobenzonitrile as a new cellulose-synthesis inhibitor. Plant and Cell Physiol. 15:389-393.

Hogsett, W.E. and R.S. Quatrano. 1975. Isolation of polysaccharides sulfated during early embryogenesis in Fucus. Plant Physiol. 55: In press.

Jaffe, L.F. 1968. Localization in the developing Fucus egg and the general role of localizing currents. Advan. Morphogenesis 7:295-328.

Kedes, L.H., P.R. Gross, G. Cognetti and A.L. Hunter. 1969. Synthesis of nuclear and chromosomal proteins on light polyribosomes during cleavage in the sea urchin embryo. J. Mol. Biol. 45:337-351.

Kinoshita, S. 1971. Heparin as a possible initiator of genomic RNA synthesis in early development of sea urchin embryos. Exp. Cell Res. 64:403-411.

Kirkwood, S. 1974. Unusual polysaccharides. Ann. Rev. Biochem. 43:401-417.

Lamport, D.T.A. 1965. The protein component of primary cell walls. Advan. Bot. Res. 2:151-218.

Larsen, G.L. and D.O. Brummond. 1974. β-(1,4)-D-glucose synthesis from UDP-($^{14}$C)-D-glucose by a solubilized enzyme from Lupinus albus. Phytochem. 13:361-365.

Ledbetter, M.C. and K.R. Porter. 1963. A "microtubule" in plant cell fine structure. J. Cell Biol. 19:239-250.

Levring, T. 1952. Remarks on the submicroscopical structure of eggs and spermatozoids of Fucus and related genera. Physiol. Plant. 5:528-539.

Lewin, R.A., editor. 1962. "Physiology and Biochemistry of Algae." Academic Press, New York.

Ley, A.C. and R.S. Quatrano. 1973. Isolation and characterization of cell walls from Fucus embryos. Biol. Bull. 145:446.

Lowry, O.H., N.J. Rosebrough, L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Maheswari, P., editor. 1973. "Recent Advances in the Embryology of Angiosperms." Catholic Press, Ranchi, India.

Mian, A.J. and E. Percival. 1973. Carbohydrates of the brown seaweeds Himanthalia lorea, Bifurcaria bifurcata, and Padina pavonia. Carbohydrate Res. 26:133-146.

Moon, A. and M. Forman. 1973. A procedure for the isolation of cell walls of Fucus furcatus embryos. Protoplasma 75:461-464.

Moore, S. 1968. Amino acid analysis. Aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. J. Biol. Chem. 243:6281-6283.

Mogensen, H.L. 1972. Fine structure and composition of the egg apparatus before and after fertilization in Quercus gambelii: The functional ovule. Amer. J. Bot. 59:931-941.

Muhlethaler, K. 1967. Ultrastructure and formation of plant cell walls. Ann. Rev. Plant Physiol. 18:1-24.

Nečas, O. 1971. Cell wall synthesis in yeast protoplasts. Bacteriol. Rev. 35:149-170.

Nelson, D.R. and L.F. Jaffe. 1973. Cells without cytoplasmic movements respond to cytocholasin. Develop. Bio. 30:206-208.

Newcomb, E.H. 1969. Plant microtubules. Ann. Rev. Plant Physiol. 20:253-288.

Neyfakh, A.A. 1971. Steps of realization of genetic information in early development. In: "Current topics in Developmental Biology," Vol. 6, pp. 45-77. Academic Press, New York.

Nisizawa, K., H. Akagawa and T. Ikawa. 1971. Dark $^{14}CO_2$-fixation in brown algae. In: "Proceedings of the Seventh International Seaweed Symposium," pp. 532-536. John Wiley and Sons, New York.

Noronha, J.M., G.H. Sheys and J.M. Buchanan. 1972. Induction of a reductive pathway for deoxyribonucleotide synthesis during early embryogenesis of the sea urchin. Proc. Natl. Acad. Sci. 69:2006-2010.

Northcote, D.H. 1969a. Fine structure of cytoplasm in relation to synthesis and secretion in plant cells. Proc. Royal Soc. B 173:21-30.

Northcote, D.H. 1969b. The synthesis and metabolic control of polysaccharides and lignin during differentiation of plant cells. Essays in Biochem. 5:89-137.

Northcote, D.H. and D.R. Lewis. 1968. Freeze-etched surfaces of membranes and organelles in the cells of pea root tips. J. Cell Sci. 3:199-206.

Novotny, A.M. and M. Forman. 1974. The relationship between changes in cell wall composition and the establishment of polarity in Fucus embryos. Develop. Biol. 40:162-173.

Percival, E. and R.H. McDowell. 1967. "Chemistry and Enzymology of Marine Algal Polysaccharides." Academic Press, New York.

Peterson, D.M. and J.G. Torrey. 1968. Amino acid incorporation in developing Fucus embryos. Plant Physiol. 43:941-947.

Pickett-Heaps, J.D. 1967. The effects of colchicine on the ultrastructure of dividing plant cells, xylem wall differentiation and distribution of cytoplasmic microtubules. Develop. Biol. 15:206-236.

Pickett-Heaps, J. 1972. Cell division in Klebsormidium subtilissimum (formerly Ulothrix subtilissima), and its possible phylogenetic significance. Cytobios 6:167-183.

Preston, R.D. 1964. Structural and mechanical aspects of plant cell walls with particular reference to synthesis and growth. In: "Formation of Wood in Forest Trees," M.H. Zimmerman, editor, pp. 169-188. Academic Press, New York.

Quatrano, R.S. 1968a. Biochemical and fine structural studies on rhizoid formation in Fucus zygotes. Ph.D. dissertation, Yale Univ., New Haven, Connecticut.

Quatrano, R.S. 1968b. Rhizoid formation in Fucus zygotes: Dependence on protein and ribonucleic acid syntheses. Science 162:468-470.

Quatrano, R.S. 1972. An ultrastructural study of the determined site of rhizoid formation in Fucus zygotes. Exp. Cell Res. 70:1-12.

Quatrano, R.S. 1973. Separation of processes associated with differentiation of two-celled Fucus embryos. Develop. Biol. 30:209-213.

Quatrano, R.S. 1974. Developmental biology: Development in marine organisms. In "Experimental Marine Biology," R.N. Mariscal, editor, pp. 303-346. Academic Press, New York.

Raff, R.A., H.V. Colot, S.E. Selvig and P.R. Gross. 1972. Oogenetic origin of messenger RNA for embryonic synthesis of microtubule proteins. Nature 235:211-214.

Ray, P.M. 1967. Radioautographic study of cell wall deposition in growing plant cells. J. Cell Biol. 35:659-674.

Ruiz-Herrera, J. and S. Bartnicki-Garcia. 1974. Synthesis of cell wall microfibrils in vitro by a "soluble" chitin synthetase from Mucor rouxii. Science 186:357-359.

Sassen, M.M.A. 1964. Fine structure of Petunia pollen grain and pollen tube. Acta Bot. Neerl. 13:175-181.

Schulz, Sr. R. and W.A. Jensen. 1968. Capsella embryogenesis: The egg, zygote and young embryo. Amer. J. Bot. 55:807-819.

Stahl, E., editor. 1969. "Thin-Layer Chromatography," 2nd ed. Springer-Verlag, New York.

Steeves, T.A. and I.M. Sussex. 1972. "Patterns in Plant Development." Prentice-Hall, Englewood Cliffs, New Jersey.

Sweeley, C.C., R. Bentley, M. Makita and W.W. Wells. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J. Amer. Chem. Soc. 85:2497-2507.

Teng, J. and R.L. Whistler. 1973. Cellulose and chitin. In: "Phytochemistry," L.P. Miller, editor, pp. 249-269. Van Nostrand Reinhold, New York.

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.

Umbreit, W.W., R.H. Burris and J.F. Stauffer. 1964. "Manometric Techniques," 4th edition. Burgess Publishing Company, Minneapolis.

Valent, B.J. and P. Albersheim. 1974. The structure of plant cell walls. V. On the binding of xyloglucan to cellulose fibers. Plant Physiol. 54:105-108.

Van der Woude, W.J., D.J. Moore and C.E. Bracker. 1971. Isolation and characterization of secretory vesicles in germinated pollen of Lilium longiflorum. J. Cell Sci. 8:331-351.

Wardlaw, C.W. 1968. "Morphogenesis in Plants." Methuen & Co., Ltd., London.

Wasserman, A. 1948. The continuous swelling of brown algae. Ann. Bot. 12:137-145.

Weisenseel, M.H. and L.F. Jaffe. 1972. Membrane potential and impedance of developing fucoid eggs. Develop. Biol. 27:555-574.

Whistler, R.L. and W.L. Wolfrom, editors. 1962. "Methods in Carbohydrate Chemistry," Vol. I. Academic Press, New York.

Whitaker, D.M. 1931. On the rate of oxygen consumption by fertilized and unfertilized eggs. I. Fucus vesiculosus. J. Gen. Physiol. 15:167-182.

Willison, J.H.M. and E.C. Cocking. 1972. The production of microfibrils at the surface of isolated tomato-fruit protoplasts. Protoplasma 75:397-403.

Wooding, F.B.P. 1968. Radioautographic and chemical studies of incorporation into sycamore vascular tissue walls. J. Cell Sci. 3:71-80.

Zonneveld, B.J.M. 1973. Inhibitory effect of 2-deoxyglucose on cell wall α-1,3-glucan synthesis and cleistothecium development in Aspergillus nidulans. Develop. Biol. 34:1-8.