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Under defined cultural conditions, zygotes of the brown alga Fucus distichus L. Powell divide synchronously to form two-celled embryos at approximately 24 hours after fertilization. These two cells differ from one another in gross morphology, ultrastructure, cytochemistry, and developmental fate. Cytochemical staining and autoradiography indicated that a sulfated polysaccharide was localized in one of the two cells. This highly polar embryo at 24 hours developed from an apolar egg, with no apparent localization of the sulfated polysaccharide. Before 10 hours, little incorporation of ^{35}S ($Na_2{}^{35}SO_4$) was detected in an acid-soluble carbohydrate fraction containing a fucan-sulfate (fucoidan). Between 10-16 hours, the time of rhizoid initiation and several hours before cell division, an increased rate of ^{35}S incorporation into fucoidan was observed. The label was bound as an ester-linked sulfate to fucoidan. Data indicated that this acidic polysaccharide was not metabolically active and was

present in a relatively unsulfated state before 10 hours. Therefore, the sulfate accumulation into fucoidan during this post-fertilization period appeared to be due to sulfation of a pre-existing polymer. Biochemical and cytochemical evidence demonstrated that sulfation of fucoidan was blocked when zygotes developed in artificial sea water containing 10 mM L-methionine instead of sulfate. Under these conditions, however, rhizoids developed normally. Thus, the process of sulfation was separated from, and appeared independent of, polar development. The degree of sulfation reached in vivo during the time of rhizoid formation was sufficient for the migration of fucoidan in an electric field on acrylamide and agarose gels. A direct correlation was demonstrated between the amount of sulfate bound to fucoidan and the rate of migration of fucoidan in an electric field. The possible role of sulfation as a mechanism for the localization of fucoidan via an electrophorectic mechanism in vivo, and the possible effect of localized fucoidan on future cell determination, were discussed.

Sulfation of Fucoidan in *Fucus* Embryos and its Possible Role in Localization

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INTRODUCTION

Overview of Problem and Rationale of System

A common feature of early embryogenesis in higher plants and animals is the development of a multicellular embryo from a highly regionalized or polar egg cell (Davidson, 1968; Balinsky, 1970; Schulz and Jensen, 1968). Determined patterns of cleavage and cell division of a polar egg cell will ultimately assure certain nuclei the inheritance of a unique cytoplasmic environment. Considerable experimental evidence has indicated that nucleo-cytoplasmic interactions exist, and that the cytoplasm can have a pronounced effect on expression of the nuclear genome and subsequent cellular differentiation.

A classic example is recorded in the work of Conklin (1905), who was studying early embryogenesis in the ascidian Styela (Cynthia). He observed that in the fertilized egg there are various pigmented areas of cytoplasm that correspond to the specific morphogenetic fates of cells inheriting these areas. Five kinds of egg cytoplasm were recognized: (1) a dark yellow cytoplasm eventually included in the tail muscles of the larva, (2) a light yellow material later segmented into the coelomic mesoderm, (3) a light gray substance

inherited by notochord and neural plate ectoderm, (4) an opaque gray material segregated into the endoderm cell lineage, and (5) a transparent cytoplasm later present only in ectodermal cells. Presumptive tissue areas appeared to develop by the partitioning of the pigmented areas of cytoplasm into the blastomeres. The complete separation of these areas into distinct cell lineages occurred by about the 64-celled stage. At this time one can recognize five distinct cell groupings based on these five types of cytoplasm. Conklin noted that the unique distribution of pigmented cytoplasm was not present to any degree in the unfertilized egg but became distributed within a few minutes following fertilization.

Hegner (1911) observed that in the zygote of a chrysomelid beetle, the germ line stem cells were derived from cells of the developing blastoderm located at one pole of the large, elongated egg. Using a hot needle, he destroyed the cytoplasm localized in this region just prior to migration of the nuclei into the periphery of the cell. These embryos would undergo apparently normal development through hatching, but the newly emerged individuals lacked germ cells. It has been well-documented that germ cells in amphibia are ultimately derived from blastomeres laid down in the vegetal hemisphere of the developing fertilized egg. Smith (1966) demonstrated that localized damage to the vegetal cytoplasm in Rana pipiens eggs by U. V. irradiation, brought about development of larvae deficient in germ

cells. Injection of some vegetal pole cytoplasm from an unirradiated egg into the irradiated vegetal pole of another egg would reverse the damaging effect allowing germ cells to form. Ultraviolet irradiation was ineffective after the 8-celled stage had been reached, indicating that the germ line stem cells were determined by this stage. It is possible that this determination occurred as early as the first cleavage which partitions one-half of the nuclei into the vegetal pole cytoplasm.

Another example where cytoplasmic injections were used to partially alter genomic function, was the "cytoplasmic corrective factor" demonstrated in the o gene mutation of an axolotl (Amblystoma mexicanum) by Briggs and Justus (1968). When females homozygous for the o gene, were crossed with normal males, embryo development was arrested at gastrulation. However, a minute amount of cytoplasm taken near the germinal vesicle of a normal egg and injected into the mutant egg, caused development to proceed beyond gastrulation and sometimes to larval stages. Based on several criteria, the corrective substance appears to be protein in nature (Briggs and Justus, 1968).

Undoubtedly, some of the most convincing evidence in support of nucleo-cytoplasmic interactions has been from investigations of "polar-lobe" development. A unique phenomenon which occurs during the early development of several protostomes, is the occurrence of a polar-lobe. During the first cleavage there is a transient extrusion

of cytoplasm which remains attached to one of the two blastomeres (CD) by a thin strand of protoplasm (trefoil stage). During completion of the first cleavage, the extruded cytoplasm flows into the CD blastomere only to reappear in the region of the developing D blastomere during the second cleavage. The lobe is reabsorbed and extruded at several subsequent cleavages, always attached to the D macromere. Wilson (1904) found that if the polar-lobe of the mollusc Dentalia was removed at the trefoil stage, the embryo failed to develop the main coelomic mesoderm band and the organs ultimately derived from it, such as the shell gland, foot, etc. Similar results were obtained from the AB blastomere which had been separated from the CD blastomere, and the isolated A, B, and C blastomeres. Evidence clearly showed that removal of the polar lobe cytoplasm, which contained no nuclear genes, resulted in the mesoderm defect(s).

More recently, Clement (1952, 1956, 1962, 1963, and 1967) has supported and expanded the work of Wilson (1904) by using Ilyanassa, the marine mollusc originally studied by Crampton, an early student of Wilson. He was able to distinguish two types of morphogenetic effects exerted by the polar-lobe cytoplasm: (1) determination of cell lineage responsible for coelomic mesoderm, and (2) determination of structures derived from other cells but inductively affected by coelomic mesoderm derivatives.

In summarizing these classical studies, determined patterns of cleavage and cell division of a polar cell will ultimately assure certain nuclei the inheritance of a unique cytoplasmic environment, and that this cytoplasm can have a pronounced effect on expression of the nuclear genome and subsequent differentiation.

Certain fundamental questions need to be asked when examining how cytoplasmic substances become localized, i. e. the establishment of cell polarity. What are the substances localized which establish polarity in these cells? How are these substances localized? How do the localized substances influence further development? The examination of mechanisms by which substances are localized has been difficult to approach experimentally. The polarization of higher animal and plant egg cells must arise sometime during oogenesis or megagametogenesis, respectively. Because these processes take place deep within the maternal tissues, it has been virtually impossible to examine the polarization mechanisms other than at a very gross, descriptive level.

Fucus is an intertidal brown alga (Division - Phaeophyta) which occurs along the northern portion of both the East and West Coasts of the United States. Unlike the eggs of most higher plants and animals, the Fucus egg appears apolar by several observable criteria (e. g. gross morphology, ultrastructure, and cytochemistry) at the time of fertilization. Shortly after fertilization, the zygote develops a

localized protruberance or rhizoid which represents the first sign of polar development. Within the cytoplasm of this localized protuberance, one can detect the development of a structural and biochemical cell polarity by the accumulation of organelles (Quatrano, 1972), sulfated polysaccharides (McCully, 1969), and RNA (Nakazawa and Takamura, 1966). The first cell division occurs in a plane which separates the rhizoidal outgrowth, and its accumulated substances, from the remainder of the zygote. The first two cells of the Fucus embryo appear different from one another in shape, size, staining properties, and organelle composition. In addition, the rhizoid cell and thallus cell will ultimately give rise to the holdfast and upright portion of the plant, respectively. Hence, the polarity exhibited by the formation of the rhizoid is the basis for cellular differentiation in the two-celled embryo, as well as for subsequent development of the organism. Unlike higher animals and plants, the Fucus zygote undergoes polar development from a previously apolar cell in the absence of maternal tissues. Since a synchronously developing population of zygotes can be readily grown in laboratory culture, the Fucus system is amenable to both a cytochemical and biochemical investigation of processes involved in cellular localization and polar development.

Purpose of the Study

Sulfated polysaccharides of animals, such as heparin and the chondroitin sulfates, have been implicated in the alteration of cell surface interactions, gene expression, and activity of specific metabolic enzymes. Sugiyama (1972) used histochemical and autoradiographic techniques to present evidence for the occurrence of a sulfated mucopolysaccharide(s) in the vegetal region of the early gastrula of sea urchin. This sulfated polysaccharide moved toward the blastocoel in conjunction with the shedding of the primary mesenchyme cells. If he blocked incorporation of sulfate into the acid mucopolysaccharide with selenate, he repressed the normal development of the archenteron (primitive gut). Sugiyama attributed control of normal cell movements during the process of gastrulation to the sulfated polysaccharide. Based on autoradiographic and biochemical data, Kosher and Searls (1973) demonstrated that in Rana pipiens, synthesis of sulfated polysaccharides occurred generally during early embryogenesis. During early gastrulation, they found little ^{35}S-sulfate incorporation into the roof of the blastocoel. However, during invagination the developing chordamesoderm incorporated considerable amounts. Only after the chordamesoderm came in contact with the overlying presumptive neural tissue, did the neural tissue begin to incorporate ^{35}S-sulfate. Kosher and Searls proposed from their data

several possible functions for the sulfated polysaccharide: (1) it might act as a primary inducer, (2) it could alter the cell surfaces for cell-cell interactions, and (3) it may participate in the cleavage process by helping to form cell-to-cell junctions.

The sulfated polysaccharide heparin has been implicated in altering the types of RNA produced by isolated nuclei of sea urchins (Kinoshita, 1971). Aoki and Koshihara (1972) presented evidence that acidic polysaccharides isolated from sea urchin cytoplasm can inhibit the activity of both E. coli and sea urchin RNA polymerases in a cell-free system. The pattern of inhibition coincided exactly with that of authentic heparin and chondroitin sulfate. Interaction of acid polysaccharide with RNA polymerase seemed to be related to an ionic interaction since certain polyanions, e.g. synthetic poly (A), exhibited similar inhibitions. If authentic heparin, chondroitin sulfate, or other acidic polysaccharide(s) from sea urchin cytoplasm were desulfated by a chemical method, they lost their inhibitory effect on the RNA polymerases. Earlier Zillig et al. (1970) found that heparin inactivated E. coli RNA polymerase by binding to the β-subunit of the enzyme.

Heparin has been shown to increase (2-3 fold over controls) endogenous DNA polymerase activity in isolated rat hepatic nuclei (Cook and Aikawa, 1973). This stimulatory effect appeared to be related to its charge characteristic since polycations, e.g. polylysine,

polyornithine and unfractionated histones, formed complexes with heparin and prevented the activation phenomenon. Based on autoradiographic evidence, new regions of the DNA were synthesized due to the heparin treatment of nuclei. Electron microscopy also revealed a dramatic change in chromatin pattern upon treatment with heparin; fiber diameters were greatly decreased, suggesting a loss of supercoiling.

Kuczenski and Mandell (1972) observed what appeared to be an allosteric activiation of tyrosine hydroxylase by heparin. They studied the effects of ionic strength, free sulfate, and heparin on the kinetics of canine hypothalamic tyrosine hydroxylase. They demonstrated that both sulfate and heparin activated tyrosine hydroxylase by increasing V_{max}. However, heparin, and not sulfate, increased the enzymes affinity for a synthetic cofactor by nearly one order of magnitude. Other mucopolysaccharides, such as chondroitin sulfate and hyaluronic acid, did not activate the enzyme.

In addition to the above studies, sulfate and sulfated polysaccharides such as heparin have been discussed with respect to their possible role in polar development and gene expression in sea urchin (Rünnstrom et al., 1964; Kinoshita, 1971) and Acetabularia (De Carli and Brachet, 1968) development. Because of the importance of sulfated polysaccharides during early embryogenesis and their possible role in regulating gene function, attention was focused on the

sulfated polysaccharides found localized in the two-celled Fucus embryo.

There is no cytochemical localization of sulfated polysaccharides in the Fucus egg or early zygote (McCully, 1970). McCully (1969, 1970) first detected the appearance of a sulfated polymer at the time of rhizoid formation. This was based on both cytochemical (Toluidine Blue O staining) and autoradiographic ($Na_2{}^{35}SO_4$) evidence. The sulfated polymer (a fucan-sulfate termed fucoidan) can initially be detected in the cytoplasm, concentrated and radiating from the side of the nucleus nearest the determined site of cell wall outgrowth. A corresponding localization of organelles and inclusions have been described by Quatrano (1972). Based on cytochemical evidence, most of the fucoidan becomes isolated within the rhizoid cell, or extracellular region of the rhizoid tip, following the first cell division of the zygote (Fulcher and McCully, 1971; Quatrano, 1968). The extracellular polysaccharide is thought to be involved with the adhesion of the two-celled embryo to the substratum. We can now ask more specific questions, that bear on the localization problem, as they relate to Fucus. What is the precise nature of the sulfated polysaccharide which becomes localized in the developing Fucus embryo? How does this sulfated polysaccharide become localized? Does the localization of this polysaccharide influence polar development, i. e. rhizoid initiation?

In attempting to test various mechanisms that might be causal in localizing macromolecules, electric currents have been demonstrated to alter the normal pattern of growth and development in many organisms. Before the turn of the century, Elfving (1882) observed that the radicles of most seedlings would orient and grow toward the cathode. Also, the orientation of seeds germinating in an electric field affected their rate of growth (Lowenherz, 1908). Schechter (1934) examined the electrical control of rhizoid formation during the regeneration of the red alga, Griffithsia bonetiana. He found that a direct galvanic current of 10-40 microamperes per square millimeter of cross-section of medium resulted in rhizoid formation at the site of the cell closest to the anode. In addition, he found that the algal filaments became graded in color, and presented evidence to show that this was not due to pH change, but to the loss of pigment from chromatophores that had moved to the anode side of the cell and "electrophoresis" of the pigment toward the cathode. Mathews (1903) was able to detect an intracellular potential difference in the hydroid Tubularia and found the polyp surface to be electronegative to the stolon surface. Using vital dyes and a naturally occurring pigment in eggs of the polychaete Nereis, Spek (1934) suggested a "cataphoretic phenomenon" underlying the migration of particles according to their electric charge.

More recently, Jaffe (1966) was able to detect an intracellular electric current developing across the Fucus zygote several hours before the appearance of the rhizoid. One possible mechaism for the localization of charged molecules, including the sulfated polysaccharide, fucoidan, could be a directed movement of this substance in response to the intracellular electric current. Jaffe (1970) has proposed that "in traversing the cytoplasm the current will generate a field that may significantly localize negatively charged molecules or particles toward the growth point (or, if there are any, positively charged ones toward its antipode)."

The purpose of this study was to: (1) identify and characterize the polymer which appears to become sulfated at the time of rhizoid formation, (2) determine whether this represents de novo synthesis, sulfation of a previously existing polymer, and/or unmasking of an already present sulfated polysaccharide, (3) determine if the localization of this sulfated polysaccharide is necessary for polar development, and (4) demonstrate whether the apparent sulfation of this macromolecule is related to its migration in an electric field.

MATERIALS AND METHODS

Preparation of Biological Material

Freshly harvested receptacles of Fucus distichus L. Powell (Yaquina Head, Newport, Oregon) were washed several times with cold tap water and blotted dry between paper towels. This procedure was repeated before the receptacles were placed in shallow trays, covered with Millipore filtered (0.45μ) sea water or artificial sea water (ASW). Both sea water mixtures were stored in the dark at 4°C. The ASW contained: 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). ASW minus sulfate was made by substituting 0.057 M $MgCl_2$ for the $MgCl_2$ and $MgSO_4$ concentrations given above. Receptacles were then placed in a lighted incubator (600 ft. c.) at 15°C for gamete release.

Since F. distichus is monoecious, fertilization occurs during, or very shortly after shedding of the gametes from the receptacles. This shedding generally takes place in the light within one hour after the inductive treatment described above. The receptacles were removed after shedding and the zygote suspension poured through a 102μ, Nitex nylon mesh. This mesh size allowed the zygotes to pass through but retained the larger debris; e.g. intact oogonia, frond fragments, etc. The filtered zygotes were washed three times with ASW (4°C) using a volume at least 100 times that of the packed cell volume for each

washing. Finally, uniform cell suspensions were pipetted into 50 x 12 mm sterile petri dishes (Falcon Plastics) for further incubation and experimental manipulation. Development occurred for 24-48 hrs, at 15°C in a lighted or darkened incubator, depending on the individual experiment.

Zygotes to be grown in ASW minus sulfate or ASW containing known amounts of sulfate were first shed from the receptacles into ASW lacking sulfate. The zygotes were then washed and distributed as above into ASW minus sulfate. If sulfate was added to the ASW during incubation, it was added to the samples as Na_2SO_4 in ASW.

Eggs of F. distichus were labeled during oogenesis by incubating washed receptacles in Millipore filtered sea water containing $NaH^{14}CO_3$ (1 μCi/ml sea water). Receptacles were placed in a lighted incubator for 60 hours at 15°C. During this period some shedding of gametes did occur. The receptacles were removed from the incubator, washed several times in cold tap water to remove shed gametes, and placed in open plastic bags to help prevent desiccation. These receptacles were stored 7-10 days at 4°C in the dark. At the end of this storage period, gametes were shed into ASW containing 10 mM sulfate according to the procedure previously described.

To label fucoidan after shedding, uniform cell suspensions were incubated for various times in the presence of exogenous $Na_2{}^{35}SO_4$ (2 μCi/ml ASW). Incorporation of labeled fucoidan precursors was

examined by incubating zygotes in the presence of ^{3}H-L-fucose (2.5 μCi/ml ASW), $NaH^{14}CO_3$ (1.0 μCi/ml ASW), or ^{14}C-D-mannose (2.5 μCi/ml ASW).

Fucoidan Isolation

Zygotes or embryos washed with ASW were homogenized in 85-90% ethanol (4°C) using 75-105 μ diameter glass beads (Sigma) and a Duall sintered glass homogenizer which was driven by an electric motor. It was determined microscopically that greater than 90% of the cells were broken by three minutes homogenization with this apparatus. Some suspensions were fractionated immediately, while others were stored for periods up to two weeks at -20°C in ethanol. The homogenate was centrifuged (10,000 g/15 min), the pellet resuspended, and washed three times in 85% ethanol.

The ethanol-insoluble pellet was resuspended in 5.0 ml of 0.25N HCl and allowed to stand for 3-4 hours at 20°C, or overnight at 4°C with occasional stirring. This suspension was centrifuged (39,000 g/20 min), and 1 volume of 95% ethanol was added to the supernatant. The resulting cloudy suspension was centrifuged (10,000 g/15 min), the pellet was discarded, and 9 volumes of 95% ethanol were added to the clear supernatant. The very fine, white suspension was centrifuged, the supernatant discarded, and the pellet (fucoidan fraction) washed two times in 85% ethanol and stored in 95% ethanol at -20°C.

To purify further, the crude fucoidan was dissolved in 5.0 ml of 50 mM citrate buffer (pH 3.0) or distilled water and kept at 4°C. While stirring, 1.0 ml of cold 3.0% (w/v) cetyltrimethylammonium-bromide (CTAB) was added, dropwise, to this fucoidan solution. The resulting precipitate was collected by centrifugation and redissolved in 5.0 ml of 2.5 M KCl. If any undissolved material remained it was discarded by centrifugation. The fucoidan-CTAB complex was reprecipitated from solution by the addition of 20 ml of distilled water (0.5 M KCl final concentration). The precipitate was collected by centrifugation and washed in 0.5 M KCl, 85% ethanol, and then stored in 95% ethanol at -20°C.

Chromatography and Electrophoresis

Purified fucoidan (20 mg) was dissolved in 2.0 ml of 1N HCl and incubated at 100°C for 7 hrs in a sealed tube. The resulting yellow solution was clarified by centrifugation, and the supernatant was treated with a mixed-bed ion exchange resin (Biorad AG 11A8). One hundred microliters of the resin-treated hydrolysate were spotted on 5 x 20 cm glass plates coated with a 250 μ layer of silica gel G and chromatographed in 3 different solvent systems: (1) n-Butanol: glacial acetic acid:diethyl ether:water (9:3:3:1); (2) n-Butanol:glacial acetic acid:water (3:1:1); (3) n-Butanol:methanol:water (5:3:1).

Developed plates were scanned on a Packard Model 7201 Radiochromatogram Scanner and then sprayed with anisaldehyde or anisidine-HCl reagents as described by Stahl (1969).

Fucoidan isolated from zygotes which had been labeled during oogonial development was hydrolyzed as above. Fifty microliters were spotted on 20 X 20 cm plates coated with silica gel (250 μ), along with glucose and fucose standards, and chromatographed in solvent system (1). The standards were sprayed with anisaldehyde which located and distinguished by color, the glucose and fucose spots. The areas corresponding to the standards were scraped from the hydrolyzed fucoidan sample for detection of radioactivity by liquid scintillation counting using a Packard Liquid Scintillation Spectrometer (Model 2425).

For electrophoresis, purified fucoidan was dissolved in 50 mM citrate buffer (pH 3.5) containing 5% (w/v) sucrose and applied (50-100 μl containing 50-100 μg of fucose) to a 5% acrylamide gel prepared according to Davis (1964), using ammonium persulfate as catalyst. Because of a distinct yellow color in the extract which migrated as fast as a marker dye (bromphenol blue), no dye was added to the samples. When the yellow band migrated off the gels (90-120 minutes at 4 mA/gel), the gels were removed from the glass tubes and stained in 1.0% acetic acid containing 0.1% toluidine blue O (TBO) for at least 2 hours at room temperature. Unbound stain was removed from gels

by leaching overnight in 7.0% acetic acid. The cleared gels were then scanned at 550 nm in a Schoeffel Microdensitometer to detect the metachromatic colored fucoidan bands. Duplicate gels not stained were sectioned with razor blades, and the slices were homogenized with distilled water in a Duall sintered-glass homogenizer. After standing for 3 hours at room temperature, aliquots of the suspensions were assayed for radioactivity while the remainder was centrifuged (20,000 g/10 min). To the supernatant, 10 volumes of cold ethanol were added and the precipitates were stored at -20°C for later analysis of fucose content. Similar procedures were used on agarose gels in which fucoidan was electrophoresed according to the method of Horner (1967).

TBO Spot Test

Aliquots (0.05-0.30 ml) of fucoidan were applied to Whatman (3 MM) filter paper disks (25 mm diameter) under a stream of warm air. The dried paper was stained for 15 minutes in 0.1% toluidine blue O (TBO) in 1% acetic acid, and destained by leaching in several changes of 7% acetic acid. Under these conditions fucoidan stains metachromatic (red) as opposed to orthochromatic (blue). The metachromatic color of the TBO-fucoidan complex was removed by shaking the disk in 25 ml of 95% ethanol containing 1% (v/v) NH_4OH.

This procedure produced a blue colored solution which was centrifuged to remove filter paper fragments and read at 620 nm in a Spectronic 20 colorimeter.

Sulfatase Assay

Equal numbers of zygotes were collected at various times during the first 24 hours of development (1-3 cc packed cells, depending on experiment) and centrifuged (2,000 g/3 min) in an International Clinical Centrifuge. The cells were resuspended in 0.1 M Tris-acetate buffer (pH 7.1) and homogenized in a Duall sintered glass homogenizer according to the procedure previously described. The homogenate was centrifuged (2,000 g/3 min) and the supernatant used for the sulfatase assay. The supernatant was incubated (37°C) in the presence of 5 mM p-nitrophenyl sulfate or 5 mM p-nitrocatechol sulfate (Sigma) for various times up to 2 hours. At the end of the incubation period, 0.1 M Tris-HCl (pH 9.8) was added and the reaction mixtures were allowed to stand 5-10 minutes before measuring the optical density at 400 nm or 515 nm respectively, in a Spectronic 20 spectrophotometer.

Determination of Rhizoid Initiation and Elongation

Rhizoid formation was scored by counting all cells lying completely within a field (100 X magnification) of a compound microscope.

Contiguous fields were counted along a line which bisected the dish along its diameter. In certain experiments, determination of nuclear division and complete cell division was facilitated by treating cells with a hematoxylin stain (Whitman, 1965) just prior to examination. Rate of rhizoid elongation was determined using measurements taken from zygotes at various times during development. Zygotes were initially selected on the basis of showing signs of rhizoid formation. Sequential measurements were taken over the 24 hour period using the same set of zygotes that were originally selected. Thus, the measurements represent data taken from a selected sample of the entire population. Measurements were obtained using an ocular micrometer at 400X magnification and were taken parallel to the long axis of the developing embryo. Since it is known that during at least the first 48 hours of development the diameter of the Fucus zygote proper does not increase appreciably (Quatrano, 1968), measurements included the entire embryo along this axis.

Radioactivity Determinations

L-Fucose-^{3}H (G) and $Na_2{}^{35}SO_4$ were obtained from New England Nuclear and had specific activities of 1.25 Ci/mmole and 850 mCi/mmole, respectively. The International Chemical and Nuclear Corporation supplied D-mannose-UL-^{14}C (spec. act. 80 mCi/mmole and $NaH^{14}CO_3$ (spec. act. 58.6 mCi/mmole). The L-amino acid-^{14}C

(U) mixture (spec. act. 57 mCi/mAtom carbon) and ^{35}S-methionine (spec. act. 323 mCi/mmole) were purchased from Amersham/Searle.

Aliquots of radioactive fucoidan from chemical fractionation or gel electrophoresis were made to 1.0 ml with distilled water and vigorously shaken in 10.0 ml of Aquasol (New England Nuclear). The clear, colorless samples were counted in a Packard Tri-Carb liquid scintillation spectrometer (Model 3310) which operated at 76% efficiency for ^{14}C, 66% for ^{35}S, and 44% for ^{3}H or Model 2425 which operated at 88% efficiency for ^{14}C, 85% for ^{35}S, and 60% for ^{3}H. Protein samples were collected on glass fiber filters (see the next section), dried and counted in a toluene based scintillation fluid (Omnifluor-New England Nuclear). Scrapings from silica gel G chromatographic separations were added directly to Aquasol (New England Nuclear), mixed and counted. All cpm reported were adjusted for background and corrected for quenching by use of an external standard.

Determination of the Effect of Sulfate Deficiency on Protein Synthesis

Zygotes, collected and prepared as previously mentioned, were distributed into 50 x 12 mm petri dishes (Falcon Plastics) containing either 0.1 M or 1.0 M sulfate and a ^{14}C-amino acid mixture (0.5 μCi/ml ASW). After various incubation periods, the zygotes were

immediately centrifuged at low speed (2,000 rpm/2 min) in a Sorvall GLC-1 and resuspended in cold 10% trichloroacetic acid (TCA). Samples were either analyzed immediately or stored at -20°C for analysis the next day. The cell suspension solution was made to 5% TCA by the addition of cold distilled water and homogenized using the method previously described. The homogenate was centrifuged (10,000 g/10 min) at 4°C. The resulting pellet was washed three times with cold 5% TCA, resuspended in 5% TCA, and heated in a 90°C water bath for 10 minutes. After centrifuging, the pellet was again washed three times with cold 5% TCA. The TCA-insoluble material was resuspended in 5% TCA and aliquots removed for determination of total protein and radioactivity.

Aliquots used for determination of radioactivity were first filtered through glass fiber filters (Whatman GF/B) using a Millipore filtering apparatus. The TCA-insoluble material was retained by the filter and subsequently washed three times with cold 80% ethanol followed by three washings with cold 95% ethanol. Filters were oven dried at 60°C for several minutes and then placed into liquid scintillation vials containing a toluene based scintillation fluid (New England Nuclear) and counted in a liquid scintillation spectrometer. The total protein was determined on separate aliquots by the procedure of Lowry et al. (1951).

Determination of the Effects of L-Methionine and L-Cysteine on Rhizoid Formation and Fucoidan Sulfation

Zygotes were allowed to develop 24-48 hours in ASW under one of the following conditions: (1) no sulfate added, (2) 10 mM sulfate or (3) 10 mM L-methionine or 10 mM L-cysteine (Sigma) and no exogenous sulfate. After the incubation period, the zygotes were scored for rhizoid formation by the previously described procedure. To determine if fucoidan was sulfated to any extent and localized in the zygotes, they were fixed overnight in 45% acetic acid and stained in a 0.1% solution of TBO containing 25 mM HCl for 3 minutes. They were then destained in 2.5 mM HCl for 5 minutes at room temperature. A similar staining procedure had been shown previously to detect sulfated fucoidan cytochemically (McCully, 1969; Quatrano and Crayton, 1973). Photomicrographs were taken using a Leitz microscope and Ektachrome film (Kodak).

Chemical Determinations

Fucose content of extracts was determined by a modification of the cysteine method of Dische and Shettles (1948) as reported by Ishihara (1964). To each 1.0 ml of fucoidan sample, 4.5 mls of cold 32N H_2SO_4 were added and vigorously stirred in an ice bath. The samples were then placed in a boiling water bath for 3 minutes and

immediately cooled to room temperature in the ice bath. After cooling to room temperature, 0.1 ml cysteine reagent (3.0% aqueous cysteine-HCl) was added while stirring the samples. After leaving the samples at room temperature for 1-2 hours, the absorbance at 396 nm and 427 nm was determined using a Spectronic 20 spectrophotometer. Standard fucose samples were run with this assay for quantitation of the unknown samples by the standard curve method. The amount of fucose (μg) was determined by the difference in absorbance between the two wavelengths (Δ O.D.) relative to the standards.

Desulfation of purified fucoidan was done according to Percival (1964) with 0.09 N HCl in methanol at room temperature. Fucoidan was labeled in vivo with $^{35}SO_4$, then isolated and purified from two-celled embryos of F. distichus. The labeled fucoidan was resuspended in methanolic-HCl and at various times aliquots of the suspension were removed and centrifuged (10,000 g/10 min). The supernatants were counted in a liquid scintillation spectrometer for released $^{35}SO_4$ and the pellets (partially desulfated fucoidan) were saved for agarose electrophoresis and the TBO spot test.

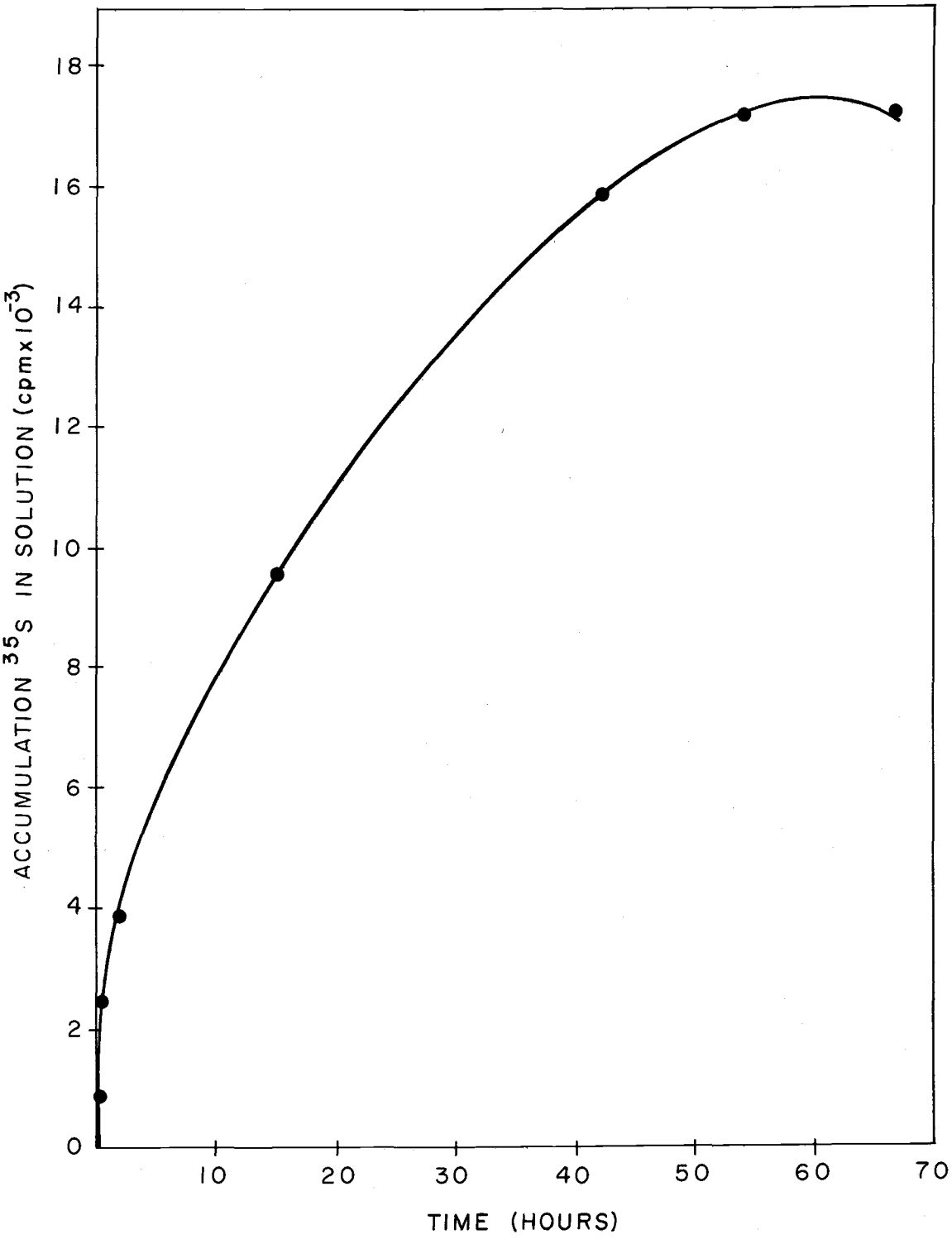
RESULTS

Sulfate Acceptor and Time of Sulfation During Embryogenesis

What is the nature of the polysaccharide being sulfated in developing Fucus zygotes (c.f. McCully, 1969)? When does this sulfation occur in relation to the period of rhizoid formation and subsequent localization of the sulfated polysaccharide in the rhizoid cell? To attempt to answer these questions, experiments were undertaken in which zygotes were pulse-labeled with $Na_2{}^{35}SO_4$ at different times during the first 24 hours of embryo development. Incorporation of ^{35}S into a crude polysaccharide fraction and subsequent fractionation and purification of this labeled polysaccharide led to its characterization and identification. Also, the temporal relationship between this sulfation and embryo morphogenesis was determined.

Zygotes were incubated in artificial sea water (ASW) with $Na_2{}^{35}SO_4$ (2μ Ci/ml) for 60 minutes at various times after fertilization. Saturation of the intracellular sulfate pool appeared complete by 60 minutes (Table 1). Approximately 15 percent of the total labeled ethanol-insoluble material was recovered as a dilute acid-soluble substance. Over 80% of the ^{35}S in this fraction was released by 0.09N HCl in methanol (Figure 1), indicating the sulfate was present in an ester-linked bond to the carbohydrate (Percival and McDowell,

Figure 1. Release of ester-linked ^{35}S from purified fucoidan isolated from two-celled embryos of F. distichus. Embryos were incubated for several hours in ASW containing $Na_2{}^{35}SO_4$ (2μ Ci/ml ASW). The labeled fucoidan was suspended in methanol containing 0.09 N HCl at 22°C, and at various times aliquots of the supernatant were counted in a liquid scintillation spectrometer to determine the rate of desulfation.


ACCUMULATION ^{35}S IN SOLUTION (cpm x 10^{-3})
0
2
4
6
8
10
12
14
16
18
10
20
30
40
50
60
70
TIME (HOURS)

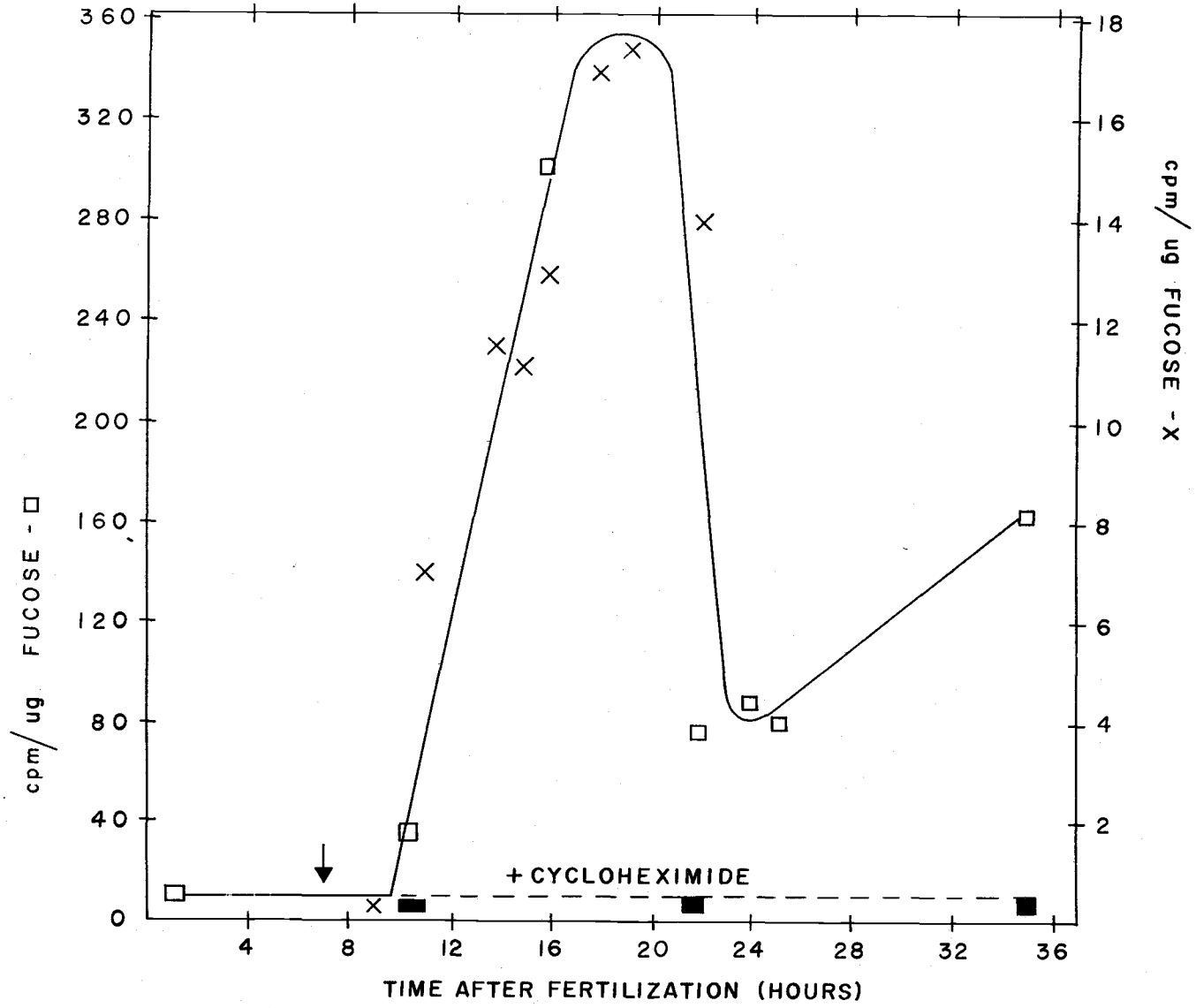
1967), rather than a more stable carbon-sulfur bond found in amino acids (proteins) and sulfolipids (Schiff and Hodson, 1973). This fraction was shown by Mian and Percival (1973) to contain 40-60% of the total "fucans" in various brown algae, including Fucus, and will be operationally defined as the fucoidan fraction.

Table 1. Accumulation of ^{35}S into a 50% ethanol-soluble fraction by equal-sized populations of intact zygotes, 15 hours after fertilization.

Total Time of $Na_2{}^{35}SO_4$ Incubation (min)	Ethanol-Soluble Fraction (cpm)
0	--
15	3,570
30	3,600
60	4,550

No incorporation of ^{35}S into this fraction was observed until approximately 10 hours after fertilization, several hours before rhizoid formation. This result was consistent with autoradiographic (McCully, 1970) and cytochemical data (Quatrano, 1972). From 10 hours, there was a steady increase in the rate of ^{35}S incorporated into the fucoidan fraction, reaching a maximum at 16-18 hours. At that time all zygotes possessed rhizoids. After 18 hours, a decrease in rate was detected (Figure 2). Since the internal pool of ^{35}S appeared to be saturated by the end of the pulse period, the differences in sulfate incorporation into fucoidan were probably not due to

Figure 2. Incorporation of ^{35}S (2 μ Ci/ml) into the fucoidan fraction of F. distichus embryos pulsed for 60 minutes at different times after fertilization. Zygotes treated with cycloheximide (0.5 μg/ml) 7 hours after fertilization did not possess rhizoids at 36 hours. The specific activity of fucoidan is expressed as cpm/μg fucose in the fucoidan fraction. The ordinates represent two different experiments having different cell populations. See Appendix I for raw data.


360
320
280
240
200
160
120
80
40
0
18
16
14
12
10
8
6
4
2
4
8
12
16
20
24
28
32
36
cpm/ ug FUCOSE - □
cpm/ ug FUCOSE - X
+ CYCLOHEXIMIDE
TIME AFTER FERTILIZATION (HOURS)

changes in sulfate permeability. This conclusion was substantiated, when the radioactivity incorporated into fucoidan was expressed as a fraction of the total counts in the soluble fraction (85-90% ethanol soluble) of the cells. Again, the same labeling pattern was observed (Figure 3).

Although the observed kinetics of fucoidan sulfation do not appear to be due to changes in the sulfate pool and/or in the permeability of zygotes to sulfate, 10-30% more sulfate entered the zygotes during rhizoid formation than at earlier times after fertilization. An interesting observation was that after a 15 or 30 minute pulse, 17 hour zygotes, unlike one hour old zygotes, were capable of increasing the amount of ^{35}S in the intracellular pool during a chase period in label-free ASW containing sulfate (Table 2). The alginic acid content of zygote cell walls increases dramatically during early development in Fucus (Moon and Forman, 1972; Ley and Quatrano, 1973). Since this polysaccharide possesses ion exchange and absorption properties which may serve as a mechanism to concentrate certain ions from sea water into the wall (Wasserman, 1948, 1949; Mongar and Wasserman, 1952), it is possible that concentration of $^{35}SO_4^{-2}$ into the wall by complexing with alginic acid during the pulse period, and its later release into the cell during the chase period in the presence of unlabeled sulfate, could account for these results in older zygotes.

Figure 3. Pattern of ^{35}S (2 μ Ci/ml) accumulation into the soluble and fucoidan fractions of F. distichus embryos pulsed for 60 minutes at different times after fertilization. Data is expressed as the ratio of counts incorporated into the fucoidan fraction over the counts found in an 85-90% ethanol extract from the same sample. Zygotes treated with cycloheximide (0.5 μg/ml) 7 hours after fertilization did not possess rhizoids at 36 hours. See Appendix I for raw data.

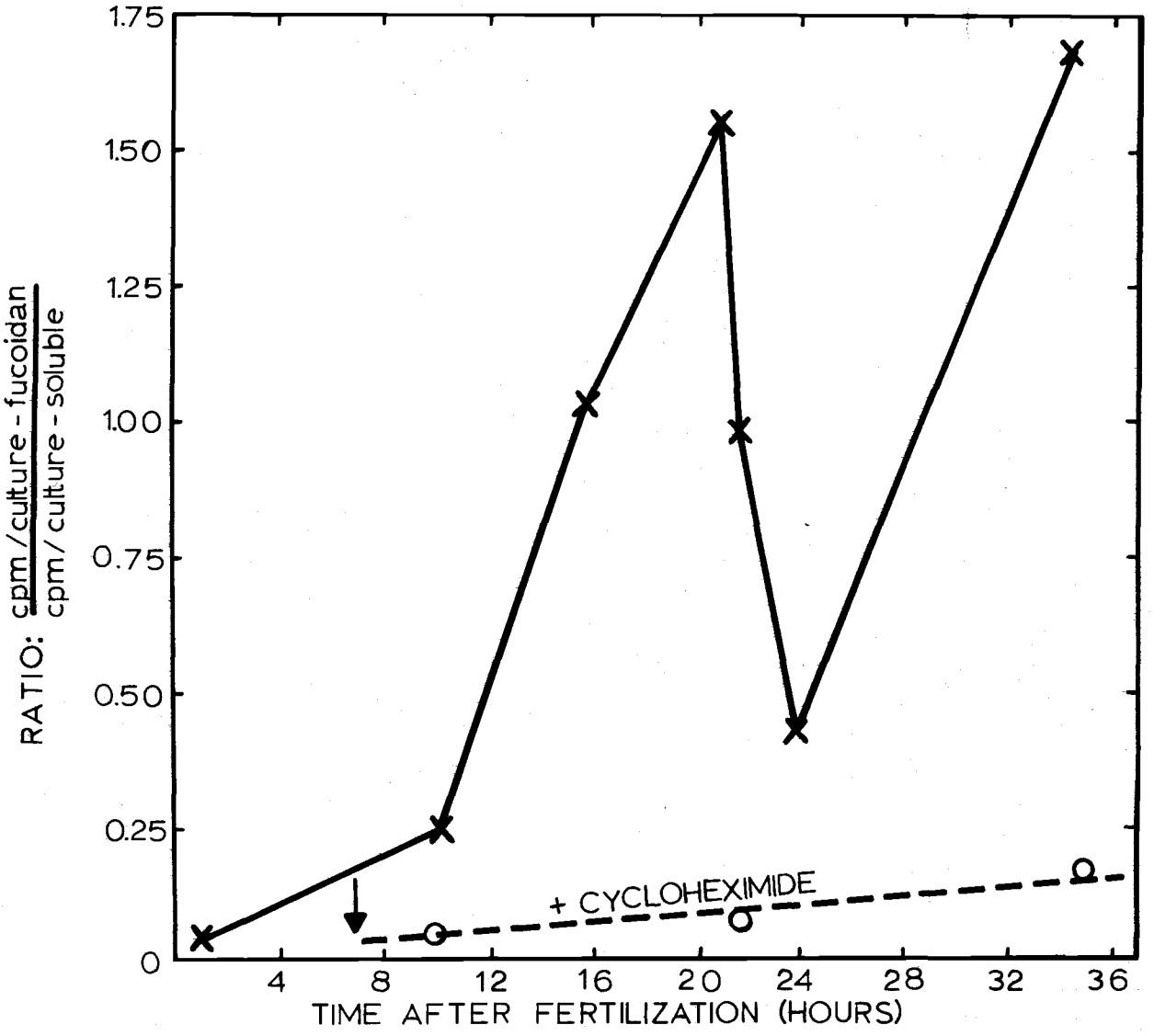

1.75
1.50
1.25
1.00
0.75
0.50
0.25
0
RATIO: cpm/culture - fucoidan / cpm/culture - soluble
+ CYCLOHEXIMIDE
4
8
12
16
20
24
28
32
36
TIME AFTER FERTILIZATION (HOURS)

Table 2. Accumulation of ^{35}S into a 50% ethanol-soluble fraction by equal-sized populations of intact zygotes with varying chase periods.

Time of Incorporation After Fertilization (hrs)	Total Time of $Na_2{}^{35}SO_4$ Incubation (min)	Time of Chase (min)	Ethanol-Soluble Fraction (cpm)
1	30	0	8,900
		15	7,920
		45	8,060
		90	7,150
17	30	0	9,530
		15	20,420
		45	16,550
		90	29,260

The sulfation of fucoidan appeared to be dependent upon protein synthesis, since cycloheximide prevented the accumulation of sulfate into fucoidan (Figures 2 and 3). Protein synthesis and rhizoid formation were inhibited by 94% in Fucus when treated in this manner with cycloheximide, and both the biochemical and morphological effects were reversible when the inhibitor was removed (Quatrano, 1968). Whether fucoidan sulfation was a necessary step in the formation of the rhizoid will be discussed later. Although the precise site of sulfation inhibition by cycloheximide could not be directly determined, the internal concentration of free sulfate was 55% less in zygotes treated with cycloheximide than in controls without cycloheximide. However,

inhibition of fucoidan sulfation was due to the effect of cycloheximide on some process in addition to uptake of sulfate (e.g. inhibition of the synthesis of sulfating enzymes), since the ratio of sulfate incorporation into fucoidan over sulfate uptake into the zygote was much lower than controls (Figure 3).

To determine the nature of the sulfate acceptor, the labeled fucoidan fraction was subjected to acrylamide gel electrophoresis. When sections of the gels were analyzed, approximately 85-90% of the fucose content and radioactivity of the applied sample migrated at the same rate in a single, broad, metachromatically stained (TBO) band (Figures 4 and 5). Commercial fucoidan (K & K Laboratories) migrated at the same rate as the fucoidan from embryos. A more detailed chemical analysis of purified fucoidan from 24 hour embryos and fronds showed the fucoidan to be a fucose polymer complexed with protein, free of other sugars and uronic acids, and containing approximately 28% sulfate by weight (Quatrano and Crayton, 1973).

Control of Fucoidan Sulfation

Earlier work suggested that fucoidan present in zygotes less than 10 hours old, contains little if any sulfate as determined by autoradiography and TBO staining properties (McCully, 1969, 1970; Quatrano, 1968, 1972). This has been substantiated more recently

Figure 4. Polyacrylamide gel electrophoresis of a fucoidan fraction isolated from 24 hour F. distichus embryos. The sample was dissolved in 50 mM citrate buffer (pH 3.5) containing 5% sucrose and applied (50-100 μl containing 50-100 μg of fucose) to a 5% acrylamide gel. Samples were electrophoresed for 120 minutes at 4 mA/gel. Gels stained with Toluidine Blue O and scanned at 550 nm exhibited a single, broad band (——). Most of the fucose (dotted bars) and the ^{35}S radioactivity (----) are associated with the stained band.

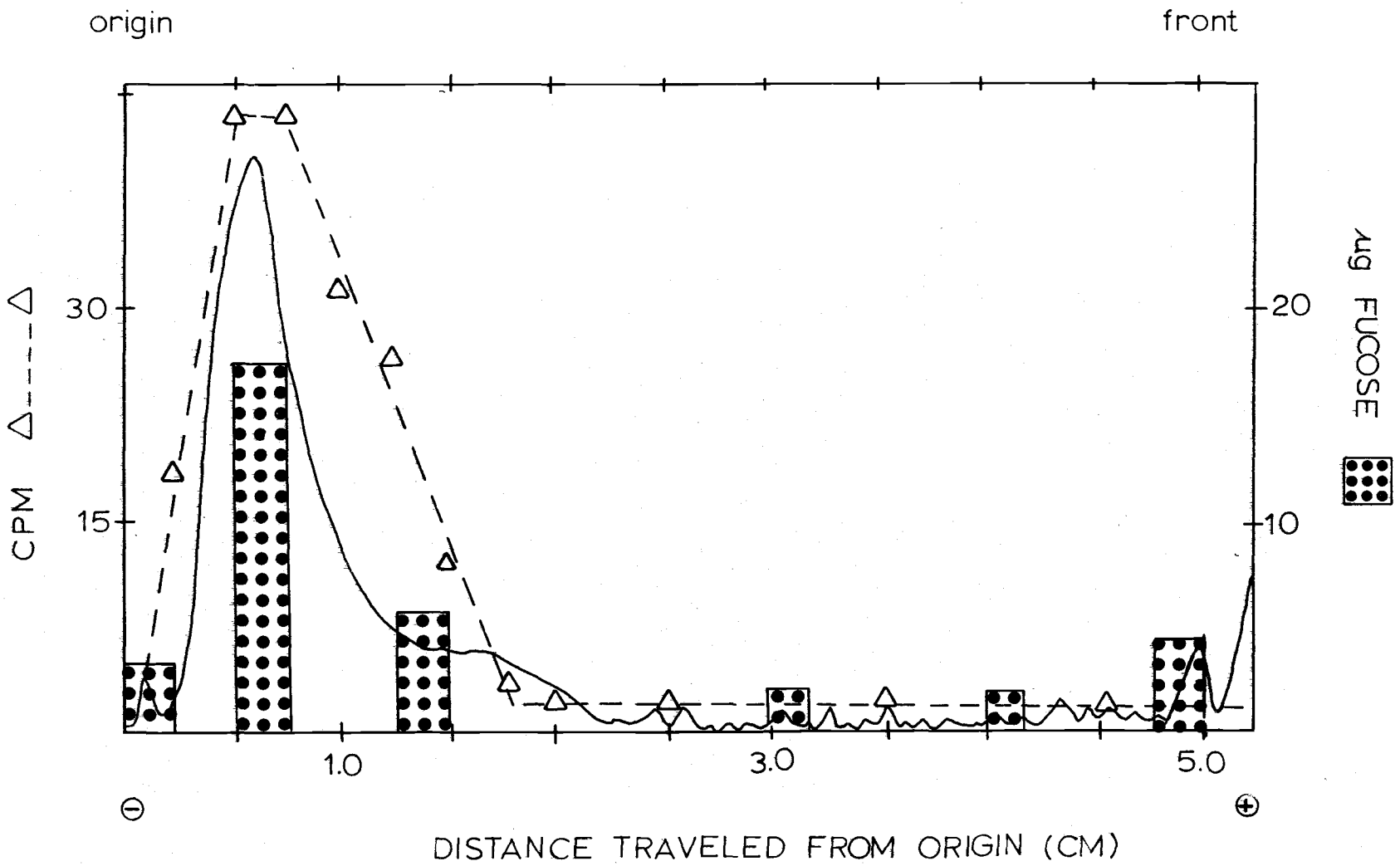
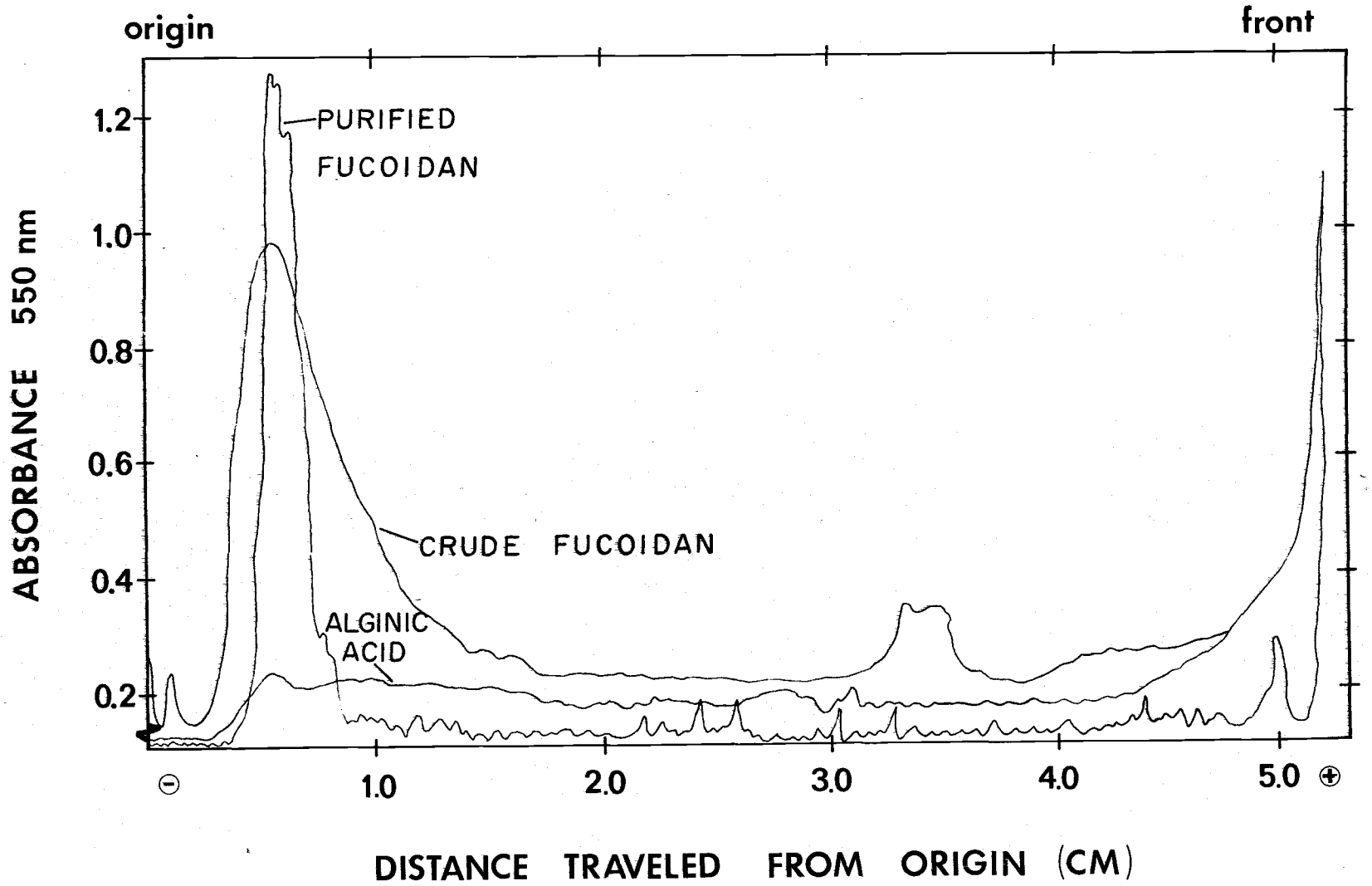

origin
front
CPM
30
15
20
10
Avg FUCOSE
1.0
3.0
5.0
⊖
⊕
DISTANCE TRAVELED FROM ORIGIN (CM)

Figure 5. A composite scan of crude fucoidan, purified fucoidan and alginic acid separated by polyacrylamide gel (5%) electrophoresis and stained with Toluidine Blue O (TBO). Each of the three samples, having identical amounts of carbohydrate, was applied to separate gels and electrophoresed for 120 min at 4 mA/gel. Gels were stained with TBO and scanned at 550 nm. Alginic acid was not observable under the staining conditions employed.


origin
front
PURIFIED
FUCOIDAN
CRUDE FUCOIDAN
ALGINIC
ACID
ABSORBANCE 550 nm
1.2
1.0
0.8
0.6
0.4
0.2
⊖
1.0
2.0
3.0
4.0
5.0 ⊕
DISTANCE TRAVELED FROM ORIGIN (CM)

by direct sulfate determination by Quatrano and Crayton (1973). They also demonstrated that fucoidan from young zygotes could not be isolated using cetyltrimethylammonium bromide (CTAB). Lack of complex formation between CTAB and fucoidan is indicative of the absence of a net negative charge on the molecule.

Although all of this data suggests that fucoidan sulfate appears in zygotes after 10 hours, the biochemical basis of this regulation remains obscure. At least four possibilities, all consistent with previous data, must be considered:

A. The unmasking of sulfated fucoidan by a basic polymer (e.g. protein), which would neither involve the synthesis of the polymer nor the sulfation of a preexisting unsulfated fucan.

B. The inhibition of active sulfatases which would result in the appearance of a stable sulfate ester on fucoidan.

C. The de novo synthesis of fucoidan from free fucose and sulfate.

D. The sulfation of a pre-existing unsulfated fucan.

Unmasking Mechanism

Kelly (1955) reported that basic proteins can bind to certain mucopolysaccharides, thereby masking their negative charges and preventing certain stains (e.g. TBO) from exhibiting a metachromatic

color. Although purified fucoidan contains some protein, the lack of a metachromatic stain prior to 10 hours does not appear to be due to the binding of basic proteins, since complete removal of proteins by various chemical treatments and specific proteases caused no change in the in vivo or in vitro staining properties of fucoidan (Quatrano and Crayton, 1973).

Sulfatase Mechanism

The rapid accumulation of sulfate into fucoidan during rhizoid initiation (Figure 1) may be due to the inhibition of sulfatase(s) present in the zygotes during early postfertilization stages. The artificial substrates, p-nitrocatechol sulfate and p-nitrophenyl sulfate, were both used to screen for the presence of active sulfatases during the first 24 hours of development. Enzyme preparations did not reveal any sulfatase activity during this time period. However, similar procedures and substrates have revealed the presence of a sulfatase in frond material from Fucus (Quatrano, personal communication).

De Novo Synthesis Mechanism

When crude fucoidan was isolated from the same number of zygotes at different postfertilization periods, the amount of fucose obtained from the fucoidan fraction did not vary (Table 3). If the total amount of fucoidan remained constant throughout the first 24

Table 3. Amount of fucoidan* present in postfertilization stages.

Expt. 1		Expt. 2		Expt. 3	
Hours after fertilization	µg fucose/ zygote	Hours after fertilization	µg fucose/ zygote	Hours after fertilization	µg fucose/ zygote
2	27	11	32	1	38
6	26	14	26	10	40
12	28	15	28	16	39
14	28	16	24	21	40
		17	28	24	38
		18	28	25	40
		19	25	34	35
		23	22		

*Amount of fucoidan expressed as micrograms (µg) of fucose released after hydrolysis of a fucoidan fraction.

hours of development, the increase of sulfate in this fraction was most likely due to a sulfation of a pre-existing polymer. However, the observed kinetics of sulfate accumulation and a constant level of fucoidan/zygote could be explained by variations in the turnover rate of fucoidan, with a relatively high rate beginning at about 10 hours. If the kinetics of sulfate accumulation into fucoidan represented simultaneous fucose polymerization and sulfate attachment, or synthesis of sulfated fucoidan by a polymerization of fucose-sulfate, incubation of the zygotes in radioactive fucose and/or metabolic precursors of fucose, should demonstrate any metabolic activity of

fucoidan. However, little if any incorporation of ^{3}H-fucose or ^{14}C-mannose was detected in fucoidan fractions from zygotes pulse labeled 3-4 hours at various times after fertilization. As seen in Table 4, both labeled compounds were taken up by the zygotes as was demonstrated by the soluble counts. However, when zygotes were labeled with $NaH^{14}CO_3$, a very large amount of radioactivity was recovered in the crude fucoidan fraction. Hydrolysis of this labeled fucoidan fraction and subsequent separation by thin-layer chromatography, demonstrated that 95% of the radioactivity was associated with a glucose component (Figure 6). This glucose probably represents contamination from the glucan laminaran (Bourne et al., 1969). Further purification of fucoidan fractions from zygotes (16-24 hours old) and fronds by precipitation with CTAB removed the glucose contaminant and yielded a preparation containing only fucose when hydrolyzed and chromatographed in three solvent systems (see Materials and Methods). Little ^{14}C label was associated with the fucose moiety of fucoidan indicating that the molecule was metabolically stable during the time of sulfation.

Sulfation Mechanism

The above data indicated that the sulfation occurring at 10 hours after fertilization was probably due to the sulfation of a pre-existing fucan which is unsulfated and metabolically quite inactive. If this

Figure 6. Tracings from a radiochromatogram of the separated sugar components found in a fucoidan hydrolyzate. Zygotes with rhizoids were incubated in $NaH^{14}CO_4$ (1.0 μCi/ml) in filtered sea water from 15 to 19 hours after fertilization. A fucoidan fraction was then isolated at 19 hours and hydrolyzed. An aliquot (100 μl) of the hydrolyzate was applied to a thin-layer chromatogram and developed in solvent (1) at room temperature. After 3 hours, the plates were dried and scanned for radioactivity at a speed of 2 cm/min, and a split width of 5 mm. They were then sprayed with anisaldehyde for detection of the sugar components.

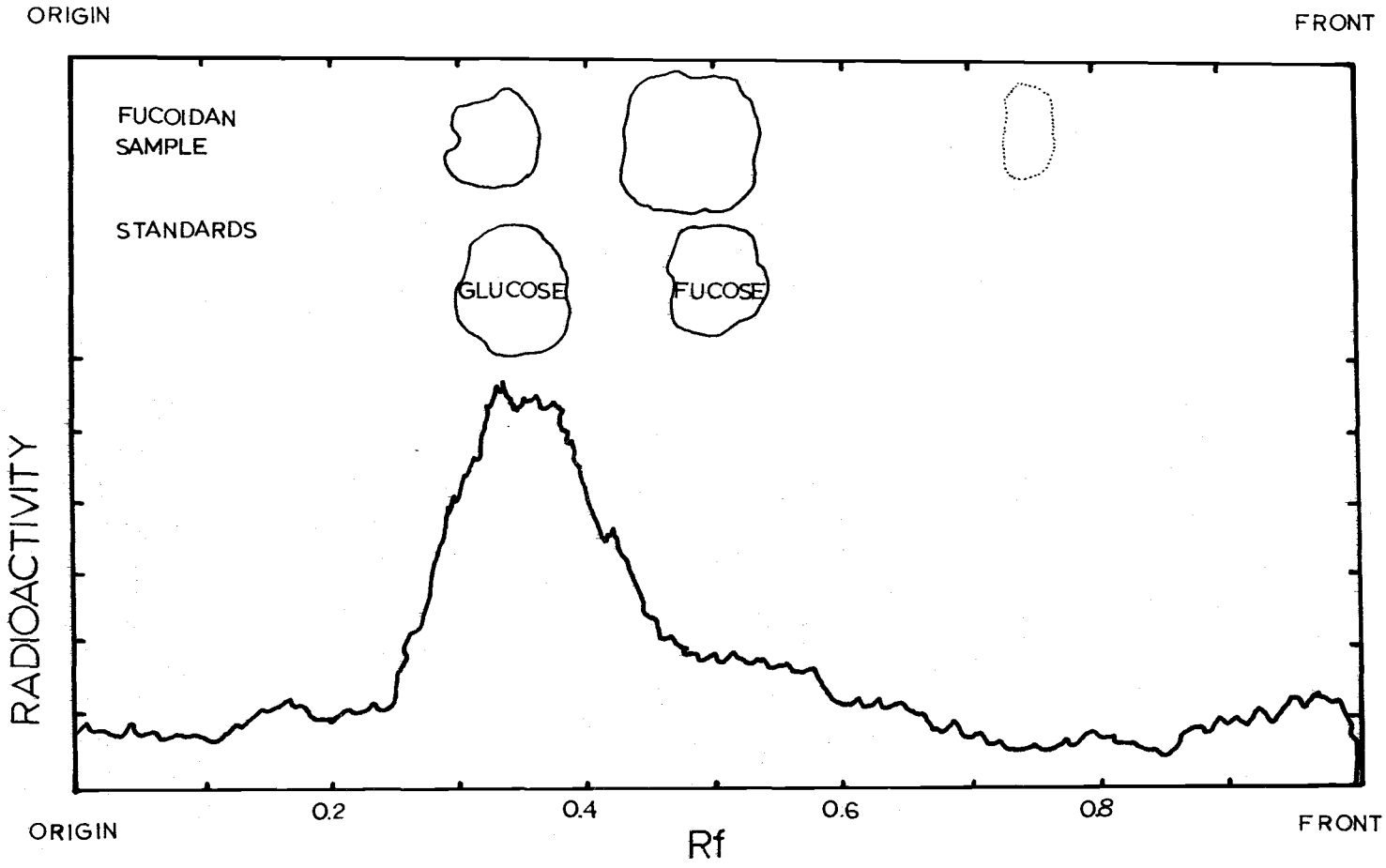

ORIGIN
FRONT
FUCOIDAN
SAMPLE
STANDARDS
GLUCOSE
FUCOSE
RADIOACTIVITY
0.2
0.4
0.6
0.8
ORIGIN
FRONT
Rf

Table 4. Incorporation of labeled fucoidan precursors into an ethanol (85%) soluble fraction and into a fucoidan fraction from postfertilization stages.

Time of pulse-label (hours after fertilization)	cpm X 10^{-3} per culture dish[a]					
	^{3}H-L-Fucose[b]		$NaH^{14}CO_3$[b]		^{14}C-D-mannose[b]	
	Soluble	Fucoidan	Soluble	Fucoidan	Soluble	Fucoidan
8-11	0.5	0.0	---		---	
9-13	---		472.6	42.6	2.6	0.6
15-19	---		455.6	53.0	4.0	0.5
16-19	0.9	0.0	---		---	
20-24	---		414.6	81.7	5.0	0.6
31-34	1.6	0.0	---		---	
32-36	---		579.1	70.0	16.6	1.7

[a] An equal number of zygotes were pipetted into each culture dish.

[b] The amounts of radioactivity present in each milliliter of culture for the three experiments were: ^{3}H-L-Fucose, 2.5 μCi; $NaH^{14}CO_3$, 1.0 μCi; ^{14}C-D-mannose, 2.5 μCi.

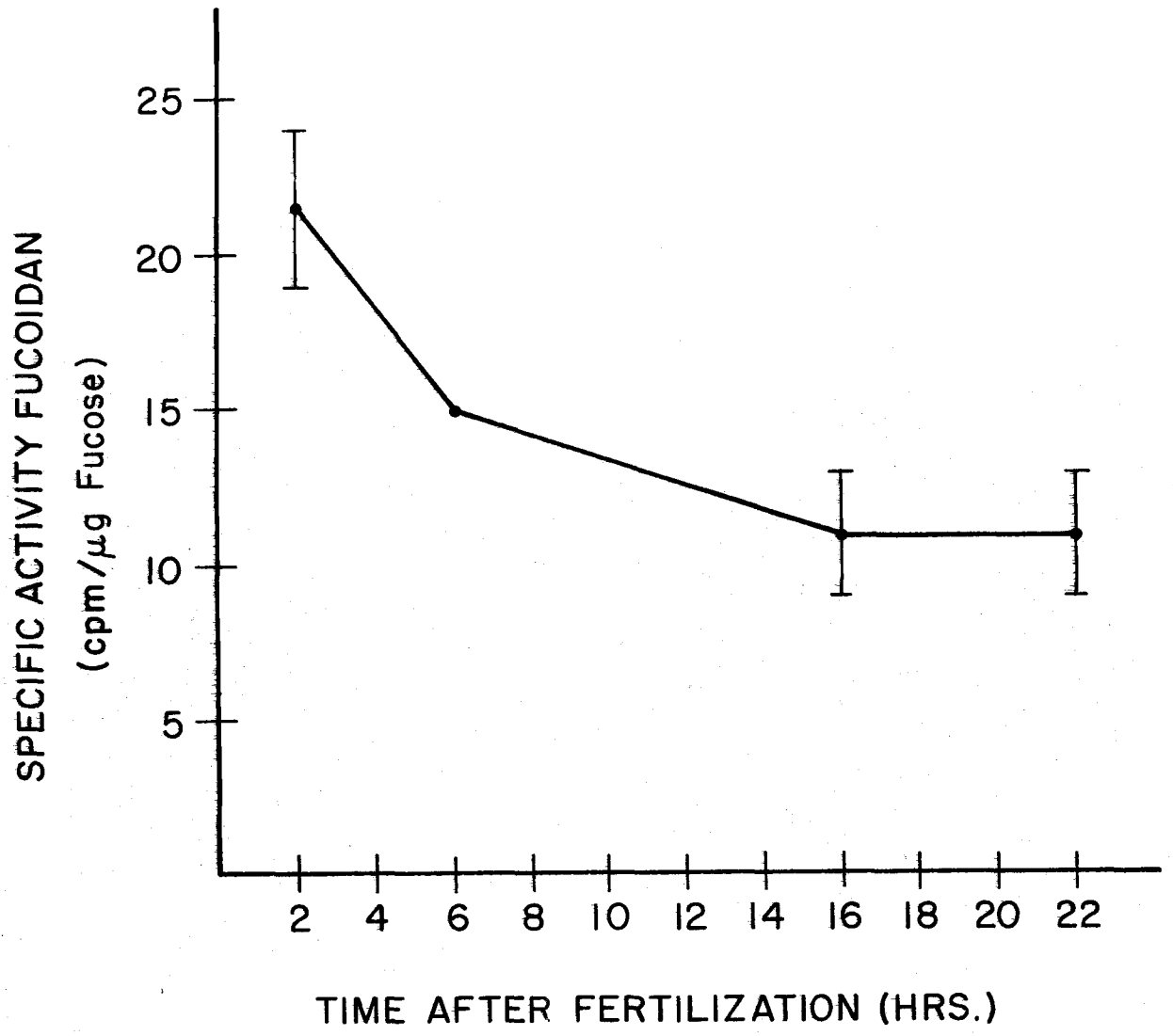
interpretation is correct, labeled fucose in fucoidan from eggs should maintain a constant specific activity during sulfation when zygotes are grown in radioactive-free ASW following fertilization.

Gametes, still within the receptables, were labeled with $NaH^{14}CO_3$ 7-10 days prior to shedding. Upon shedding, the fucoidan isolated from eggs of F. vesiculosus or young zygotes of F. distichus was labeled with ^{14}C. Labeled eggs were fertilized and incubated in ASW without radioactive sulfate. Fucoidan was then isolated from zygotes at various times after fertilization, hydrolyzed, separated by thin-layer chromatography and the specific activity (cpm/μg fucose) of the fucose moiety of fucoidan determined (Figure 7). There was a pronounced decrease in specific activity between 0-6 hours which then maintained a constant level up to 22 hours. If the sulfation kinetics were to be explained by de novo synthesis of the fucoidan, the accumulated activity in the fucoidan would be expected to decrease rapidly at about 10-18 hours. It appears, therefore, that the above results are consistent with the idea that the control of sulfate accumulation into fucoidan resides at the level of sulfation of a previously synthesized fucan.

Relationship Between Sulfation of Fucoidan and Rhizoid Initiation

Does fucoidan sulfation play a direct, causal role in the initiation of polar growth in developing Fucus embryos? The initial

Figure 7. Changes in the specific activity of radioactively labeled fucoidan isolated from F. distichus zygotes at various times after fertilization. The fucoidan in eggs was labeled by incubating the receptacles in $NaH^{14}CO_3$ (1 μCi/ml sea water) 7-10 days prior to shedding. Shedding of the gametes, subsequent fertilization and development took place in ASW lacking ^{14}C.


SPECIFIC ACTIVITY FUCOIDAN
(cpm/μg Fucose)
25
20
15
10
5
2
4
6
8
10
12
14
16
18
20
22
TIME AFTER FERTILIZATION (HRS.)

approach to this question was to determine if polar growth (i. e. rhizoid formation) occurred in zygotes grown in ASW minus sulfate, a condition which should prevent fucoidan sulfation.

Only 10% of zygotes of F. distichus grown in ASW minus sulfate formed rhizoids (Table 5). However, nuclear division occurred and was only slightly inhibited compared to controls (Table 5). The small percentage of rhizoids formed was probably due to utilization of endogenous sulfate pools, since eggs of F. vesiculosis (a dioecious species), incubated in ASW minus sulfate for several hours before fertilization, exhibited less than 5% germination (Crayton et al., 1974). When zygotes were allowed to develop in ASW containing 25-250 μM inorganic sulfate, the proportion of the zygote population developing rhizoids appeared to be directly related to the amount of sulfate present in the medium (Figure 8). It was determined that 50% germination occurred at 75-100 μM sulfate, while concentrations above 250 μM resulted in germination comparable to controls. It also appeared that the rate of rhizoid elongation was directly related to the amount of exogenous sulfate. If a concentration of sulfate was given that permitted rhizoid formation in 50% of the population (0.1 mM), the elongation rate of those forming rhizoids was 1.7 μ/hour as compared to 3.2 μ/hour in ASW containing 10 mM sulfate (Figure 9). Therefore, when sulfate was limiting, the rate of polar growth was

Figure 8. Relationship between rhizoid formation in F. distichus and inorganic sulfate concentration in the medium. Zygotes were allowed to develop for 24-48 hours in ASW containing various concentrations of sulfate (added as Na_2SO_4). At the end of the incubation period, zygotes were scored for rhizoid formation by counting contiguous fields along a line which bisected the dish along its diameter.

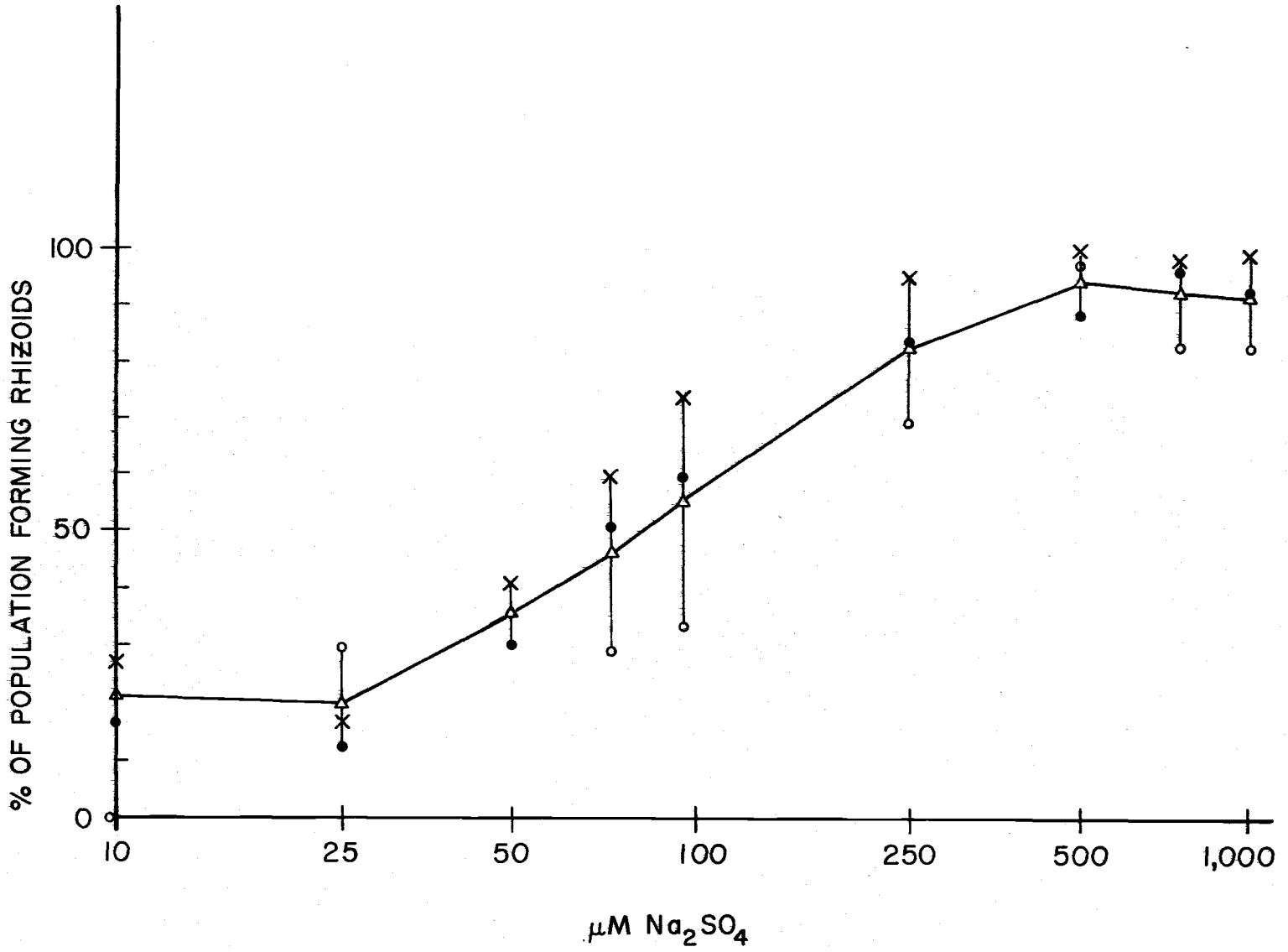
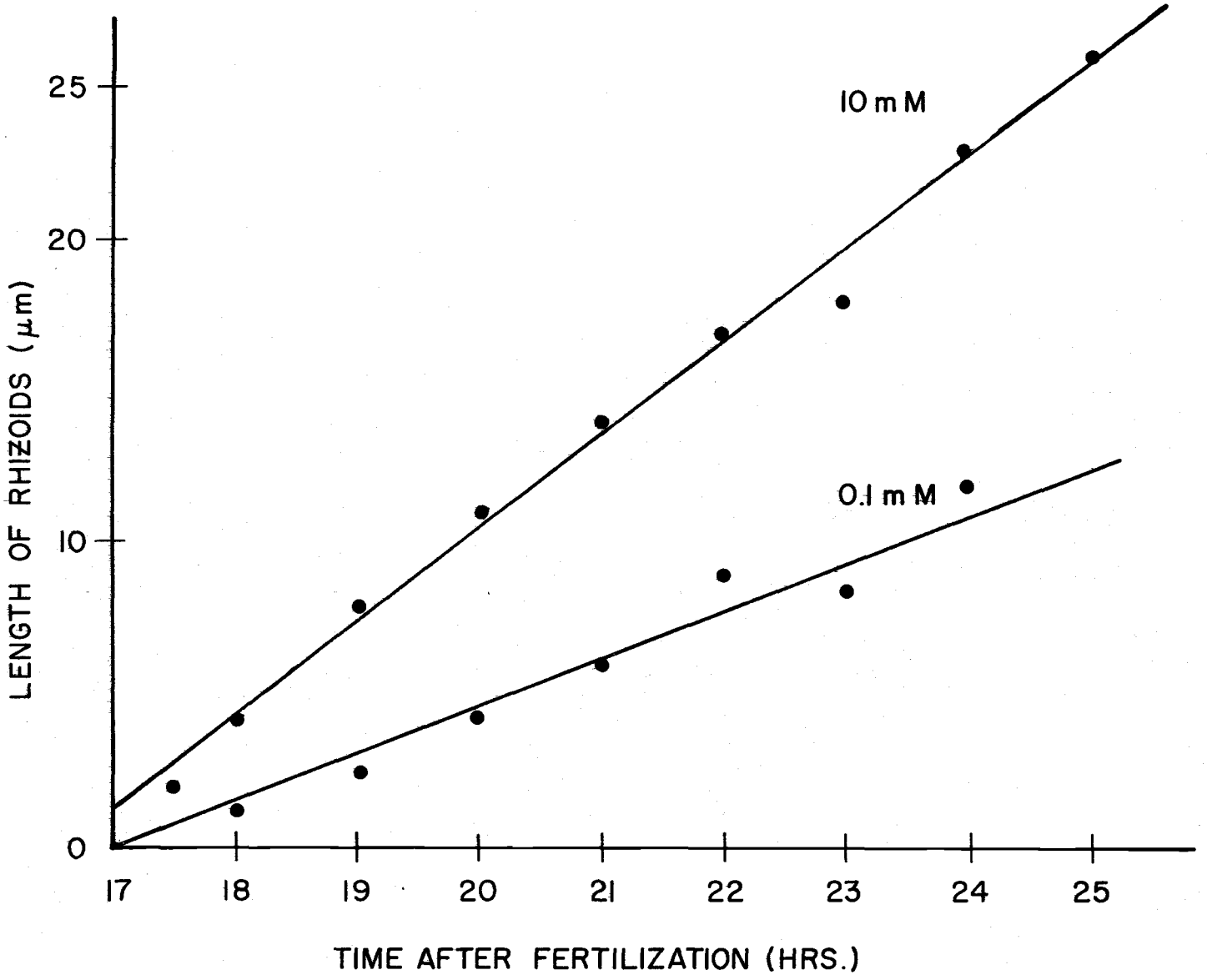

% OF POPULATION FORMING RHIZOIDS
100
50
0
10
25
50
100
250
500
1,000
μM Na_2SO_4

Figure 9. Rate of rhizoid elongation as a result of exogenous sulfate concentration. Zygotes developed in ASW containing 10 mM or 0.1 mM sulfate and the rate of rhizoid elongation determined. Measurements were obtained using an ocular micrometer at 400X magnification and were taken parallel to the long axis of the developing embryo. Zygotes were initially selected on the basis of showing signs of rhizoid formation. Sequential measurements were taken using the same set of zygotes, thus data is representative of a selected sample of the entire population.


25
20
10
0
LENGTH OF RHIZOIDS (μm)
10 m M
0.1 m M
17
18
19
20
21
22
23
24
25
TIME AFTER FERTILIZATION (HRS.)

correspondingly reduced, and when sulfate was absent, rhizoids did not form.

Table 5. Effects of different sulfur sources on specific morphological and cytochemical characteristics of 2-celled embryos.

Additions to - SO_4 Medium	% Population Forming Rhizoids[a]	% Population Nuclear Division[b]	TBO Stain[c]
-	10 ± 2.5	87	negative
+1 mM SO_4	94 ± 3.8	99	positive
+ 10 mM methionine	88 ± 4.2	--	negative
+ 10 mM cysteine	89 ± 5.4	--	negative

[a] Each figure (± S. D.) is an average from at least 3 experiments and in each experiment greater than 200 zygotes were counted in each sample. Populations were counted 36-48 hours after fertilization.

[b] Samples counted at 24 hours.

[c] Whole embryos (24 hours) were fixed overnight in 45% acetic acid and stained in a 0.1% solution of toluidine blue O (TBO) containing 25 mM HCl for 3 minutes. They were then destained in 2.5 mM HCl for 5 minutes at room temperature. A positive reaction indicated by a metachromatic stain (pink) which is specific, under these conditions, for sulfated polymers such as fucoidan. A negative reaction indicated that only the orthochromatic stain (blue) was observed.

Quatrano (1968) reported that post-fertilization protein synthesis was essential for rhizoid formation. Peterson and Torrey (1968) demonstrated that protein synthesis occurred in two distinct phases; the first between 1-6 hours after fertilization, and the second, a more gradual increase which corresponded to the time of rhizoid formation and the first cell division. Could the absence of sulfate be inhibiting

protein synthesis, and thereby arresting polar growth, rather than preventing sulfation of fucoidan?

When zygotes were incubated in ASW minus sulfate containing a ^{14}C-amino acid mixture, incorporation into protein was found to be inhibited by 52 $\pm$ 4.5% as compared to zygotes grown in ASW (Table 6). Protein synthesis appeared to be little affected before 6 hours (Experiments 2 and 3 - Table 6), i.e. during the initial increase of postfertilization protein synthesis. However, there was a marked reduction in the accumulation of radioactivity into protein between 6-24 hours, which would correspond to the time of the second increase in protein synthesis occurring during rhizoid formation. This latter period of protein synthesis has been shown to be necessary for normal development of embryos, i.e. rhizoid formation and cell division (Quatrano, 1968). Sulfur-containing amino-acids were then added to prevent this inhibition without having exogenous free sulfate present. When L-methionine (10 mM) was added to ASW minus sulfate, rhizoid initiation was restored (Table 5). Similar results were obtained with L-cysteine. It therefore appeared that rhizoid formation occurred in the absence of exogenous sulfate providing a sulfur-containing amino acid was present. It has also been demonstrated that after isolating a protein and fucoidan fraction from 18 hour zygotes, which had been incubated in ^{35}S-methionine, no inhibition of methionine uptake into protein by zygotes in ASW minus sulfate was observed.

Table 6. Effects of exogenous sulfate (Na_2SO_4) on protein synthesis in developing zygotes as detected by ^{14}C-amino acid incorporation.

EXPERIMENT #1			EXPERIMENT #2			EXPERIMENT #3		
Time After Fertilization[a] (hrs)	Na_2SO_4 (mM)	Incorporation Into Protein[b] (cpm)	Time After Fertilization[a] (hrs)	Na_2SO_4 (mM)	Incorporation Into Protein[b] (cpm)	Time After Fertilization[a] (hrs)	Na_2SO_4 (mM)	Incorporation Into Protein[b] (cpm)
1-12	0.0	2,790	3-6	1.0	557	1-6	1.0	677
1-18	0.0	3,920	3-6	0.1	630	1-6	0.1	657
1-18	10.0	11,085	15-22	1.0	4,352	1-16	1.0	8,387
			15-22	0.1	2,200	1-16	0.1	3,730
			1-22	1.0	6,365	1-24	1.0	37,835
			1-22	0.1	4,800	1-24	0.1	14,132

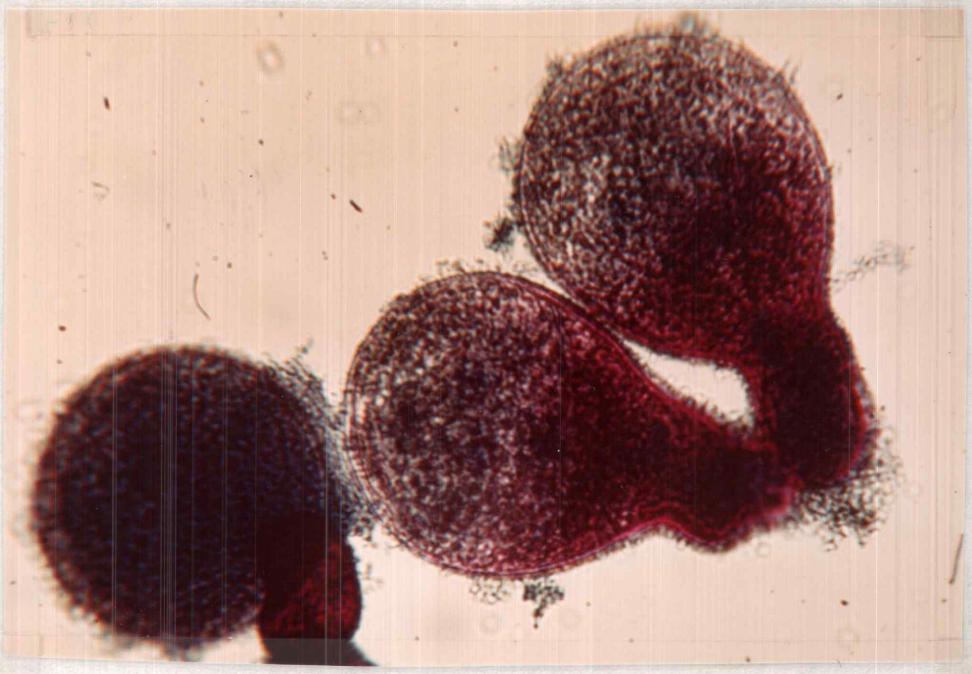
[a]This represents the time after fertilization of labeling with a ^{14}C-amino acid mixture. See Materials and Methods for details.

[b]Protein isolated from zygotes at the end of the labeling period.

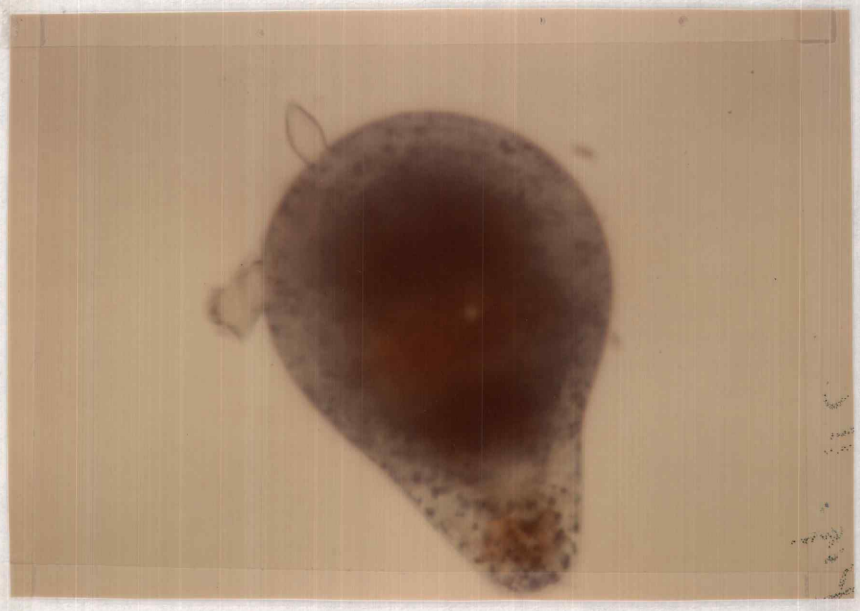
Furthermore, less than 10% of the ^{35}S from methionine found in an ethanol insoluble fraction was incorporated into fucoidan (Crayton et al., 1974). An equivalent amount of sulfur in the form of $Na_2{}^{35}SO_4$ would result in about 50 times the incorporation into fucoidan compared with ^{35}S-methionine (Quatrano and Crayton, 1973). Therefore, protein synthesis was not inhibited in ASW lacking sulfate containing methionine, and the ^{35}S from methionine was not utilized to sulfate fucoidan in the absence of Na_2SO_4.

As previously mentioned, zygotes stained with TBO in strong acid displayed a localized metachromatic staining material in the region of the developing rhizoid (McCully, 1969; 1970). Under these conditions the stain was shown to be specific for fucoidan. Zygotes grown in ASW containing 10 mM sulfate showed cytologically a similar localized metachromasy, both intracellularly and extracellularly (Figure 10A). However, zygotes grown in ASW minus sulfate, or ASW minus sulfate containing methionine, did not exhibit the presence of metachromatic material (Figure 10B), even though rhizoid formation comparable to controls was observed in the latter medium. This lack of sulfation determined cytochemically, has been recently substantiated biochemically by a direct determination of sulfate in fucoidan isolated from zygotes grown in ASW and ASW minus sulfate containing methionine (Crayton et al., 1974). Therefore, fucoidan

Figure 10. A. Embryos grown 24 hours in ASW containing 10 mM sulfate and stained with TBO. A localized metachromasy, both intracellular and extracellular, can be observed in the region of the rhizoid. Note lack of metachromatic stain in zygote that had not formed a rhizoid (bottom left). B. Zygotes grown in ASW -SO_4 containing 10 mM L-methionine and stained. Rhizoid formation was comparable to (A) but without the occurrence of detectable metachromasy. TBO staining procedure was the same for both A and B, as described in Table 5.

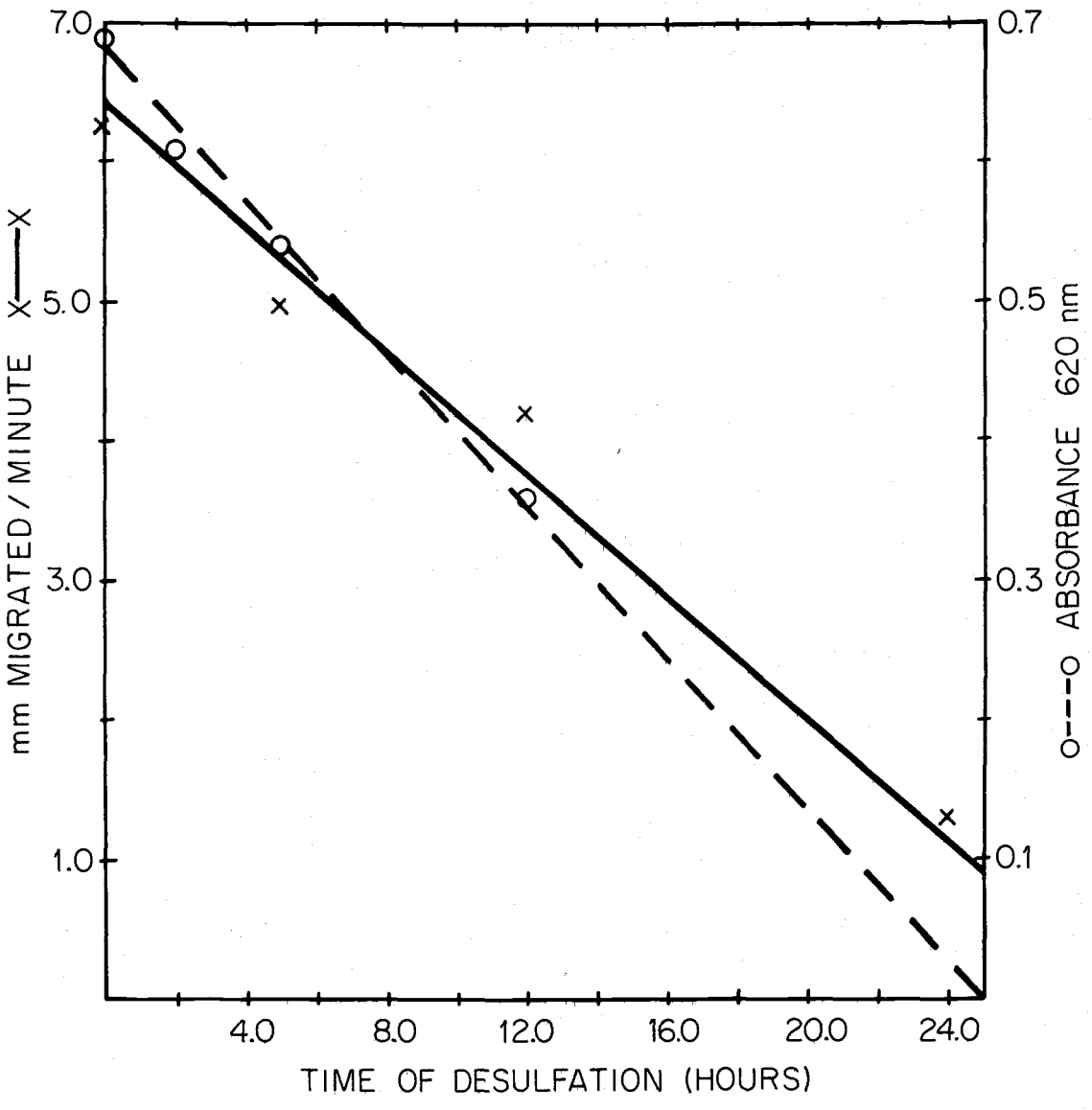


A



B

Figure 11. Relationship between the rate of fucoidan movement in an electric field (X——X) and stainability in the TBO spot test (O----O), to the sulfate content of fucoidan. Aliquots of fucoidan at different sulfate levels (i.e., at different times during chemical desulfation) were subjected to agarose electrophoresis and the TBO spot test according to techniques previously described in Materials and Methods. Approximately 80% of the initial radioactivity of fucoidan is lost during this 24-hour desulfation.


7.0
5.0
3.0
1.0
0.7
0.5
0.3
0.1
mm MIGRATED / MINUTE X—X
O---O ABSORBANCE 620 nm
4.0
8.0
12.0
16.0
20.0
24.0
TIME OF DESULFATION (HOURS)

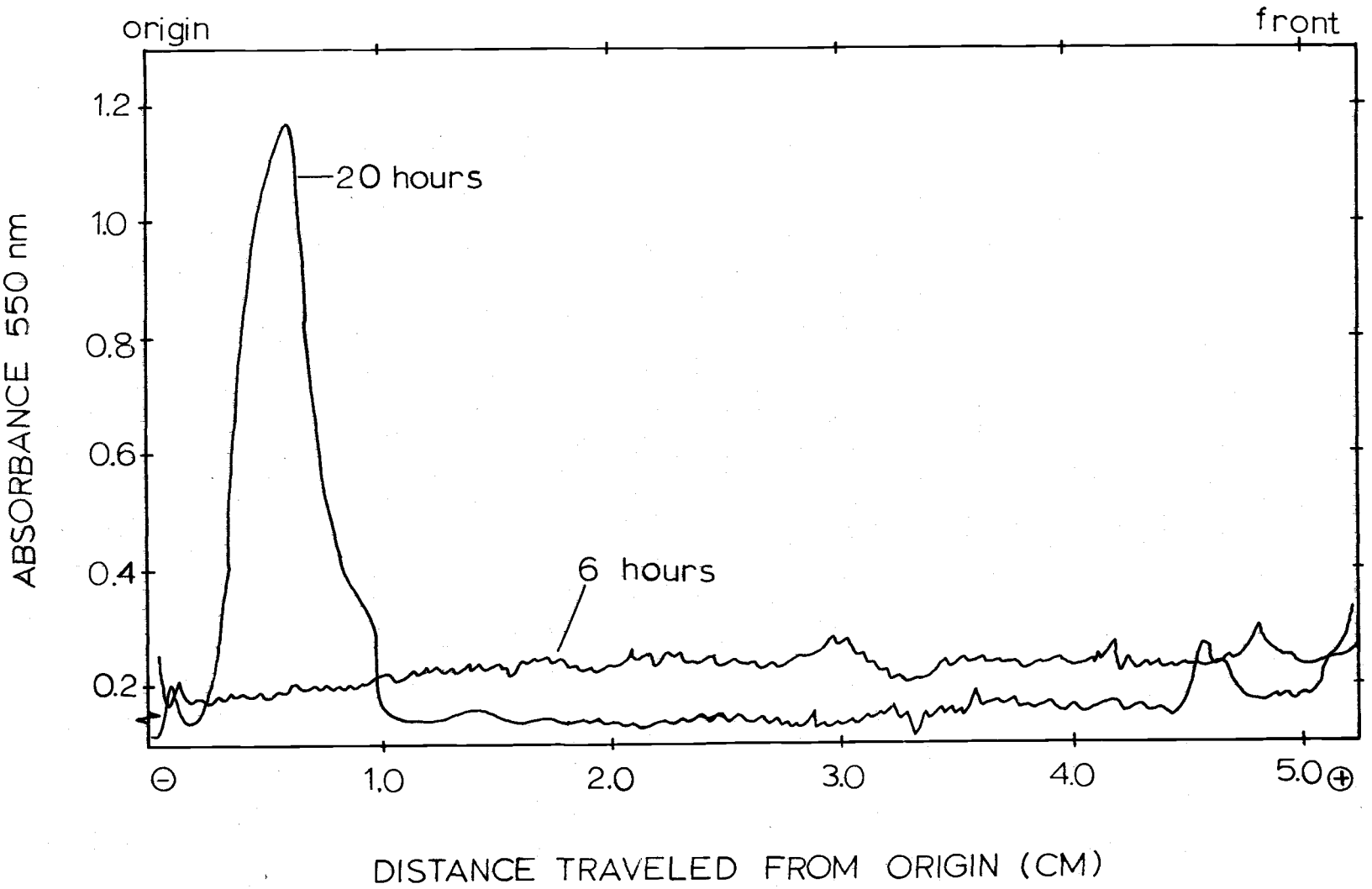
sulfation did not occur in ASW minus sulfate with 10 mM methionine, although normal polar growth was observed. It appears from this data, that sulfation of fucoidan is not necessary for polar growth.

Relationship Between Sulfation and Localization by Cellular Electrophoresis

Although sulfation of fucoidan appears not to have a causal role in initiating polar growth, it may be involved with the localization of sulfated fucoidan in the rhizoid cell by a self-electrophoresis mechanism as proposed by Jaffe (1966). To determine whether the sulfation of fucoidan could account for its migration in an electric field, fucoidan containing various amounts of sulfate was subjected to agarose gel electrophoresis. Migration in agarose gels is proportional only to charge on the molecule (Horner, 1967). Samples of fucoidan containing varying amounts of sulfate were obtained by treating ^{35}S-labeled fucoidan from two-celled embryos with methanolic-HCl which chemically removes the ester sulfate. At different times during the desulfation procedure, samples of differing sulfate content were collected for electrophoresis. The sulfate content and migration in agarose gels were determined for each sample and represented in Figure 11. It is apparent that the TBO methachromasy and the migration rate in an electric field were directly proportional to the amount of sulfate attached to the fucan polymer. When fucoidan isolated from

6 hour and 20 hour zygotes was subjected to agarose gel electrophoresis, only the fucoidan from 20 hour zygotes entered the gel (Figure 12). The above evidence indicated that sulfation of fucoidan at the time of rhizoid formation resulted in a negatively-charged molecule which could migrate in vitro in an electric field. This lends support to the hypothesis that localization of this polyanion toward the rhizoid (anode of the endogenous current described by Jaffe), may be caused by the negative charge on the polymer resulting from sulfation.

Figure 12. Tracings from two polyacrylamide gels of fucoidan prepared from 6-hr and 20-hr zygotes. Gels were stained with TBO and scanned at 550 nm. No metachromatic staining band or fucose polymer was observed in the gel containing fucoidan isolated from young zygotes. Similar results were obtained when the same samples were subjected to agarose gel electrophoresis. The samples applied were from the same number of zygotes, possessed the same amount of fucose, and were of equal volume.


origin
front
1.2
1.0
0.8
0.6
0.4
0.2
20 hours
6 hours
ABSORBANCE 550 nm
⊖
1.0
2.0
3.0
4.0
5.0⊕
DISTANCE TRAVELED FROM ORIGIN (CM)

DISCUSSION

The present study indicates that zygotes of Fucus distichus incorporated only trace amounts of sulfate into fucoidan during the first 10 hours following fertilization. Although zygotes took up appreciable amounts of sulfate during the 10 hour period, little was incorporated into fucoidan. Beginning at about 10-12 hours, several hours before rhizoid formation, the rate of sulfate incorporation into fucoidan began a significant and steady increase. Within a very short time, this rate in zygotes was sufficient to achieve a level of sulfation in fucoidan comparable to fucoidan isolated from mature fronds (Quatrano and Crayton, 1973). The sulfated polysaccharide isolated from zygotes yielded only fucose upon hydrolysis, with no detectable xylose, galactose, or uronic acids. It was precipitable with CTAB and co-electrophoresed in acrylamide gels with authentic fucoidan. Both commercial fucoidan and fucoidan from embryos migrated as a single, broad band in acrylamide or agarose gels. Based on this evidence, as well as the infra-red spectrum and solubility properties in ethanol-$MgCl_2$ (Quatrano and Crayton, 1973), the sulfated polysaccharide isolated from F. distichus embryos was fucoidan, not a glucuronoxylofucan, alginic acid, or a glucan (c.f. Larsen et al., 1970; Percival, 1971).

Bidwell et al. (1972) characterized three sulfated polysaccharides isolated from frond tips of F. vesiculosus which were metabolically active and were heterogeneous polymers containing various amounts of xylose, galactose, uronic acids, fucose, and ester-linked sulfate. They tentatively suggested from labeling patterns that the biosynthesis of "fucoidan" occurred via two heteropolymer precursors, a xylogalactofucoglucuronan and xyloglucuronogalactofucan. The results presented in this study using F. distichus embryos, indicated that only one sulfated polysaccharide was isolated from a fraction similar to that described by Bidwell et al. (1972). This sulfated polysaccharide was not metabolically active and appeared to be principally a fucan, i. e. a homogeneous polymer of fucose.

The timing of ^{35}S accumulation into fucoidan was consistent with autoradiographic and cytochemical data of McCully (1969, 1970; Fulcher and McCully, 1971). Furthermore, this sulfate was in fact incorporated into a polysaccharide which was characterized as fucoidan. The increased sulfation accounted for the localized metachromasy which was detected cytochemically during rhizoid formation. Although Kelly (1955) demonstrated that basic proteins such as protamine sulfate and histones could suppress the metachromatic properties of various mucopolysaccharides (e.g. chondroitin sulfate and heparin) when stained with TBO, protease treatments of fucoidan

isolated from zygotes at various times after fertilization did not alter the TBO staining properties.

Evidence has accumulated from various animal systems demonstrating differences concerning the mechanism of sulfate incorporation into various polysaccharides. The sulfation of chondroitin sulfate apparently occurs concomitant with chain elongation (Telser et al., 1966; Meezan and Davidson, 1967; Derge and Davidson, 1972). Conversely, sulfation of heparin has been shown to be at least partly due to sulfation of a pre-existing polymer (Lindahl et al., 1972; Robinson, 1969). Enzymatic preparations of sulfotransferases from hen uterus (Johnson and Baker, 1973) and ox lung (Foley and Baker, 1973) were able to transfer sulfate to the N- sulfate position of desulfated heparin, but not to heparin.

When fucoidan was isolated from zygotes at various times during development, it was found that the total amounts were relatively constant throughout the first 24 hours of development. Thus, it would appear that the pattern of sulfate incorporation into fucoidan would be due to the sulfation of a pre-existing fucan. Zygotes incubated for 4 hours in ^{14}C labeled CO_2, fucose, or mannose showed a low rate of turnover in the fucose component of the ethanol-insoluble fucoidan fraction at the time of fucoidan sulfation. This low turnover rate was substantiated by labeling fucoidan in eggs and following the loss of specific activity of fucoidan in zygotes after fertilization. Although

the specific activity of fucoidan remained relatively constant during sulfation, a significant decrease in the specific activity occurred between 0-6 hrs. Acidic polysaccharides have been identified as cell wall components in Chlorella (White and Barber, 1972), Dictyota (Evans and Holligan, 1973), and Fucus zygotes (Ley and Quatrano, 1973). Ley and Quatrano have shown that the newly synthesized cell wall of zygotes 20 minutes after fertilization was 60% by weight uronic acid polymers (alginic acid) and 40% cellulose with only a trace amount of fucoidan. However, between 0-6 hours the amount of fucoidan in the cell wall increased to 20% by weight and then remained constant. They attributed the appearance of fucoidan in the cell wall to de novo synthesis of fucoidan. The fucose units which appear in the cell wall could arise from a soluble pool of fucose which was not labeled during oogenesis, and account for the decrease in specific activity of the total fucoidan. However, the possibility also exists that the fucoidan in the cell wall was not synthesized de novo but came from pre-existing fucoidan not labeled during oogenesis which migrated into the cell wall. The migration of sulfated polysaccharides into the extracellular environment has been well documented (Godman and Lane, 1964; Lane et al., 1964; Evans et al., 1973). In either case, the observation of Ley and Quatrano could account for the sharp decrease in specific activity reported between 0-6 hours in this study. A low

turnover rate after 6 hours was consistent with the observations of Bidwell and Ghosh (1963), who found that when sterile frond tips of F. vesiculosus were incubated in $Na_2{}^{35}SO_4$ and $NaH^{14}CO_3$, the rate of sulfate incorporation was much greater (20-600 times, depending on duration of experiment), than that of CO_2 incorporation in fucoidan. They concluded that the sulfate component of fucoidan was rapidly exchanged without complete breakdown and resynthesis of the molecule, i. e. the carbon "backbone" or fucose moieties.

The amount of sulfate needed in the external medium for fucoidan sulfation and normal development was considerably lower than the sulfate levels in sea water. It was found that rhizoid formation comparable to controls would occur in ASW containing only 250 μM inorganic sulfate. Sea water contains approximately 25-28 mM sulfate (Spector, 1956, Harvey, 1963). This is 100 times the minimal amount needed to allow normal rhizoid formation. Thus, it would appear that Fucus zygotes are quite efficient in utilizing sulfate from sea water during the initial stages of development, and that substantial fluctuations could occur in sea water with little or no apparent effect on embryo development.

In the absence of SO_4, rhizoid formation was prevented because of the inhibition of essential protein synthesis occurring between 9-15 hours (Quatrano, 1968). Peterson and Torrey (1968) demonstrated that protein synthesis in developing Fucus zygotes occurred in two distinct

phases; the first between 1-6 hours after fertilization, and the second at the time of rhizoid formation. The inhibition of protein synthesis caused by lack of sulfate was manifested in the second phase of protein synthesis, i. e. at the time of rhizoid formation. This evidence suggests that the pool of sulfur-containing amino acids, which is critical for this protein synthesis, was depleted during the first phase of synthesis. It appears that the zygotes did not have a sufficiently large sulfate pool, or efficient means of diverting sulfur or sulfur-containing compounds into amino acid formation, to overcome the deficiency of external sulfate during this critical period of development. As a result, the second phase of protein synthesis did not occur and rhizoids were not formed. Not only was rhizoid formation dependent upon exogenous sulfate concentration, but also the rate of elongation of a rhizoid once it was initiated.

Since sulfation of fucoidan commences at about 10 hours, a few hours before the intracellular electric current observed by Jaffe (1966), is the electric current observed by Jaffe a cause or effect of the localized fucoidan? Fucoidan could become localized in the rhizoidal region by some mechanism of localized sulfation and serve as a fixed anionic gel which immobilizes incoming cations at the rhizoid tip. Under this condition, the localized polyanion could set up a fixed-charge gradient, thus an electric field, and give rise directly to the endogenous electric current which could subsequently

localize other charged molecules and initiate polar growth. Tachibana et al. (1973) located a sulfated polysaccharide in the tectorial membrane of the guinea pig by electron microscope histochemistry. They proposed that the location of the sulfated polysaccharide would have a "polarizing effect between the positive potential in the endolymph and the negative potential in the organ of Corti."

One way of testing whether sulfation of fucoidan was causal in initiating polarity would be to prevent sulfation and observe the effects on polar growth. L-methionine (10 mM) added to ASW lacking sulfate, restored rhizoid formation and protein synthesis to that of control levels (Crayton et al., 1974). Since only trace amounts of the sulfur from methionine was incorporated into fucoidan (Crayton et al., 1974), the sulfur from methionine was not utilized to sulfate fucoidan in the absence of Na_2SO_4. The absence of fucoidan sulfation in the presence of methionine was shown both biochemically and cytochemically. Zygotes forming rhizoids in ASW minus sulfate with 10 mM methionine added did not show the appearance or localization of a metachromatic material when stained with TBO. Evidence thus far presented indicated that fucoidan sulfation is not a prerequisite for events which may establish polar growth, e.g. intracellular electric current, in developing Fucus zygotes.

Lund (1921) demonstrated in animals that the direction of normal differentiation could be altered by imposing an electric field equal to the bioelectric field. He further showed that Fucus zygotes allowed to develop in an electric field would produce rhizoids on the positive side (Lund, 1923). Spek (1934), working with eggs of the polychaete Nereis, proposed a "cataphoretic phenomenon" underlying the migration of particles according to their electric charge. Endogenous bioelectric fields have been measured in such diverse structures as roots (Scott and Martin, 1962), fungal hyphae (Slayman and Slayman, 1962), and fish melanophores and iridocytes (Kinoshita, 1963). More recently, Rose (1970a, b) working with Tubularia has presented evidence showing that not only does an electrical polarity determine the polarity of regeneration by controlling the movement of charged repressors, but also, that a bioelectric field of sufficient strength is necessary for regeneration to occur. A mechanism for directed, intracellular movement of macromolecules and cellular particles in developing Fucus zygotes has been proposed by Jaffe (1966, 1970). Jaffe has measured an intracellular electric current forming at the time of rhizoid initiation which makes the rhizoid tip electropositive and suggests, according to his calculations, that it could develop sufficient strength to localize negatively charged molecules and particles at the site of rhizoid formation. Although fucoidan sulfation does not appear to be necessary for polar growth, it could give the

molecule a sufficient negative charge to enable its migration and localization at the site of the rhizoid protuberance. Evidence presented in this study has shown that the sulfation of fucoidan beginning at about 10 hours after fertilization, was sufficient to allow migration in vitro toward the positive pole of an electric field. Cytochemical and autoradiographic evidence is consistent with this mechanism occurring in vivo. However, this is only indirect evidence that such a mechanism for localizations is in fact taking place in Fucus. In vivo experiments similar to those of Lund (1923), Schechter (1934), and Rose (1970a) must be performed to substantiate the in vitro evidence presented in this study.

Once localized the fucoidan might provide "cytoplasmic feedback" to the rhizoid nucleus allowing for specific genomic expression. It should be recalled that at the two-celled stage the cells are already determined to form specific adult structures, i.e. the thallus and holdfast regions. Nucleo-cytoplasmic interactions have been documented for a wide variety of cell and organisms (Gurdon and Brown, 1965; Gurdon, 1969; Davidson, 1968). The technique of cell fusion (Harris, 1970) has brought about some dramatic demonstrations of cytoplasmic influence upon nuclear gene activity. Bolund et al. (1969) were able to produce heterokaryons from HeLa cells and chicken erythrocytes. Prior to fusion, the erythrocyte nucleus was not engaged in DNA synthesis. After fusion, they observed a

seven-fold increase in dry mass (as measured by interference microscopy), presumably due to uptake of cytoplasmic proteins. Concomitant with this increase in dry mass and volume, the erythrocyte nucleus initiated DNA synthesis. Similar results have been obtained with nuclear transplantation experiments (reviewed in Gurdon, 1967). Nuclei removed from differentiated cells of amphibia, (e.g. intestinal epithelium or brain), and transplanted into enucleated, activated eggs, began to undergo nucleic acid metabolism unlike that of the differentiated state but quite characteristic of a fertilized egg nucleus. Scheintaub and Fiel (1973) used a small angle light scattering technique to show physical alterations (including nuclear swelling) in isolated mouse spleen nuclei when subjected to heparin or dextran sulfate. No such effect was observed with chondroitin sulfate. Heparin has been implicated in altering the genomic RNA pattern from isolated nuclei of sea urchin (Kinoshita, 1971). Does fucoidan exhibit similar effects on isolated Fucus nuclei? Can fucoidan alter the activity of certain enzymes as heparin has been shown to do (Cook and Aikawa, 1973; Aoki and Koshihara, 1972; Kuczenski and Mandell, 1972)?

Although it has not been shown directly that sulfated polysaccharides can move across the nuclear membrane, evidence does exist for the movement of proteins into nuclei. Arms (1968) and Merriam (1969) demonstrated that in nuclear transplantation experiments with amphibian cells that concomitant with "shifting" of nuclear genome to

that characteristic for the recipient cytoplasm there was an uptake of proteins into the nucleus from the cytoplasm. Ringertz et al (1971) supported the earlier proposal of Bolund et al. (1969) that the nuclear swelling resulting from heterokaryon formation between HeLa cells and chicken erythrocytes coincided with uptake of cytoplasmic proteins.

A class of high molecular weight cytoplasmic proteins (greater than 65,000 daltons) isolated from Chinese hamster liver, kidney, and tissue culture cells has been described by Vaughan and Comings (1973). Using DNA-affinity chromatography to isolate the proteins, they found a certain degree of sequence specific binding to native DNA. Furthermore, the proteins showed organ specificity and bound more readily to double stranded rather than single stranded DNA. Schumm et al. (1973) have presented evidence supporting the controlled release of messenger RNA from the nucleus by cytoplasmic proteins. Based on DEAE-cellulose fractionation of cytosol proteins, they isolated two fractions which suggested that control results from both a positive and negative feedback from the cytoplasm. Competition-hybridization experiments on RNA transport in cell-free systems indicated that the proteins in one or both classes differed qualitatively in normal, regenerating, and neoplastic liver.

The intracellular site of polysaccharide sulfation has been reported in certain animal (Goodman and Lane, 1964; Lane et al., 1964; Kimata et al., 1971) and plant cells (Evans et al., 1973; Ramus and Groves, 1972; Evans and Holligan, 1973). The site appears associated with the vesicular component of the Golgi apparatus. Jaffe (1968) and Quatrano (1972) have reported the accumulation of mitochondria and Golgi in the rhizoid cell of Fucus. In addition, Golgi derived vesicles which become localized around the nucleus at the time of rhizoid formation were ultimately localized in the rhizoid cell (Quatrano, 1972). It would be interesting to see if these Golgi and derived vesicles are, in fact, the sites responsible for fucoidan sulfation beginning at about 10 hours following fertilization.

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APPENDIX

APPENDIX I

Time After Fertilization (hrs)	Specific Activity of Fucoidan (cpm/μg Fucose)	Ethanol-Soluble Fraction (cpm)	Ratio $\left(\frac{\text{spec. act. fucoidan}}{\text{ethanol-soluble}}\right)$
1	8.0	2,760	0.04
11	21.3	1,224	0.28
11 (+C)	(1.8)	(552)	(0.05)
16	306.1	4,812	0.99
22	75.9	1,464	0.83
22 (+C)	(4.7)	(564)	(0.15)
24	90.7	1,572	0.87
25	80.6	1,368	0.47
35	180.0	1,572	1.60
35 (+C)	(3.4)	(252)	(0.24)
40	392.6	1,524	4.38

(+C) = cycloheximide added.