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Enzymatic Characterization of the Morphogen Recognized by *Agaricia humilis* (Scleractinian Coral) Larvae

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Abstract. Larvae of the common Caribbean scleractinian coral, *Agaricia humilis,* are induced to settle and metamorphose by contact with specific crustose (nongeniculate) coralline red algae. This requirement for an exogenous trigger of settlement and metamorphosis has been shown to control the distribution of recruits of this coral in the natural environment. Results reported here demonstrate that the stringency and specificity of this larval requirement persist for at least 30 days following the planktonic release of the brooded larvae, thus enhancing both the capacity for dispersal of the larvae and the substratum specificity of their metamorphosis and recruitment.

The inducer of metamorphosis is shown to be associated with an insoluble macromolecular carbohydrate. This molecule is found with the partially purified cell walls obtained from a morphogenetic crustose red alga, *Hydrolithon boergesenii,* or its associated microflora. Because two non-inductive crustose red algal species also lack the cell wall-associated inducer, the substratum specificity of metamorphosis is probably the result of larval recognition of this molecule. In procedures that should prove widely applicable to other systems, purified and highly specific enzymes were used to cleave the inductive cell wall-associated polysaccharides and to solubilize the active morphogen. Enzymes were also used as probes with which to identify essential structural features required for the morphogenetic activity. These enzymatic and related biochemical studies show that the morphogen is associated with, and may itself contain, a sulfated glycosaminoglycan that includes multiple N-acetylglucosamine and galactose residues. The larval receptors that recognize this complex carbohydrate cue may thus be related to lectins. The solubilized morphogen induces normal settlement, attachment, and the metamorphosis of *A. humilis* and *A. tenuifolia* larvae on clean polystyrene surfaces, and the larvae seem to have no other requirement. This effect is apparently specific for larvae of species induced to settle by the intact alga; larvae of the sympatric coral *Tubastraea aurea* are not induced by this chemical, or by the intact algal surface. A wide variety of other natural and synthetic sulfated polysaccharides and related polymers have little or no inductive effect on the *A. humilis* larvae, suggesting that the larval receptors involved in substratum recognition are highly specific. A similar high specificity of lectin- and sulfated polysaccharide-mediated recognition, and the resulting control of differentiation, has been observed in a wide variety of biological systems.

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Abbreviations: Tris, tris-hydroxymethylaminomethane; DEAE, diethylaminoethyl; HPLC, high-pressure liquid chromatography; CCA, crustose (nongeniculate) coralline red alga/algae.

Introduction

Agaricia humilis is a shallow-water scleractinian coral common throughout much of the central and southern Caribbean. *A. humilis* releases copious planula larvae throughout the year (van Moorsel, 1983). Recruitment of this and closely related congeneric species dominates patterns of scleractinian recruitment in many areas within their range (Dustan, 1977; Bak and Engel, 1979; Rylaarsdam, 1983; Rogers *et al.,* 1984; Hughes, 1985; Hughes and Jackson, 1985; van Moorsel, 1989).

The recruitment of *Agaricia humilis* is determined, in part, by larval selection of specific settlement sites, and is thus not a wholly stochastic or lottery-like process (Morse *et al.,* 1988; van Moorsel, 1989). The substratum-speci-

ficity of these larvae is governed by their recognition, upon contact, of particular nongeniculate encrusting coralline red algae; the principal factor controlling this recognition is a non-diffusing chemical cue associated with the algal surface (Morse *et al.,* 1988). The molecule responsible is insoluble and apparently associated with the cell walls of the recruiting algae or their microbial symbionts (Morse *et al.,* 1988). Instability of the molecule has further complicated efforts to characterize the inducer.

To overcome these difficulties, we have taken advantage of the specificities of purified enzymes in analyzing and solubilizing the morphogen recognized by *Agaricia humilis* larvae. The results reported here demonstrate that the molecule, which is biologically specific in its action as a morphogen, is a sulfated glycosaminoglycan. This result further suggests that the larval receptors involved in the recognition of this inducer may be related to lectins.

Materials and Methods

Larvae, and assays of metamorphosis

The procedures used are minor modifications of those described previously (Morse *et al.,* 1988). Adult colonies of *Agaricia humilis, A. tenuifolia,* and *A. agaricites agaricites* were identified by the taxonomic criteria of Wells (1973) and van Moorsel (1983, 1989). Colonies of these species and of the sympatric ahermatype, *Tubastraea aurea,* were collected from depths of 1–5 m in Bonaire, Netherlands Antilles, and incubated in the dark in 20–60 l of seawater at 28°C. Planula larvae (0.4–2 mm in length, depending on species; van Moorsel, 1983) were released copiously between 1800 and 2300 h, collected from the surface with a Pasteur pipet, and maintained at densities of ≤0.5 larva/ml in 600 ml polystyrene containers of 0.2 μm-filtered seawater containing 2 μg/ml of the antibacterial antibiotic, rifampicin, at 28°C, under continuous indirect illumination from a 40-W incandescent bulb at 3 m.

Assays for the induction of larval attachment and metamorphosis were (unless otherwise noted) performed in duplicate samples of 5 larvae/sample, in 10 ml of seawater (0.2 μm-filtered) containing 2 μg/ml rifampicin and other additions as noted, in 20 ml polystyrene disposable breakers incubated at 28°C with indirect illumination (as described above) for 24 h. Results (except those in Table V) are presented as the mean percentage ± S.D. of larvae metamorphosed; where no deviation is shown, there was no variation between duplicate assays. Metamorphosis, defined as the differentiation and calcification of the septal ridges following permanent attachment and cellular differentiation, was quantified by examination with a binocular microscope. This transition is thus an unequivocally recognized (and in these species, irreversible; *cf.* Morse *et al.,* 1988) developmental event, and not simply a behavioral change. Under the conditions of the assay employed, metamorphosis of the agariciid larvae is density-independent and unaffected by the presence or absence of newly metamorphosed conspecifics (Morse *et al.,* 1988).

For the analysis of DEAE binding of the inducer (presented in Table V), a scale for quantitation of units of inducer activity was employed. Units are defined in the section describing the DEAE binding experiments.

Coralline red algae, and preparation of cell wall-associated morphogen

Agaricia humilis larvae can be induced to metamorphose by only certain specific nongeniculate coralline red algae (Morse *et al.,* 1988; A. Morse and R. Steneck, in prep.). Where responsiveness to such algae was measured, small, repeatedly rinsed chips (approx. 50/assay, *ca.* 1 mm^3) of an inductive species, *Hydrolithon boergesenii,* or the non-inductive species *Neogoniolithon megacarpum* and *Porolithon pachydermum* (A. Morse and R. Steneck, in prep.), collected from the habitat of the adult *A. humilis,* were used. All crustose red algae were carefully cleaned of macroscopic epibionts before use in these and all other experiments.

Preparation of the decalcified, cell wall-associated morphogen from *Hydrolithon boergesenii* (and its associated microbial symbionts) was performed at 28°C as follows. The fresh coralline alga was cleaned of macroscopic epibionts, rinsed with distilled water, scraped from its substratum, blotted dry, and ground with a mortar and pestle to a viscous paste. For each 1 g of the resulting homogenate, 10 ml of 0.2 μm-filtered seawater containing 2 μg/ml rifampicin was added, and homogenization was continued with the mortar and pestle until an extremely viscous and very finely ground consistency was achieved. This material was forced by compressed air (10 p.s.i.) through a nitrocellulose filter (0.2 μm, 47 mm diam.) in a Nalgene filter apparatus. The resulting filter cake was washed successively with 5–7 portions (20 ml each) of rifampicin-containing seawater, the filtrand being resuspended as necessary, until the last filtrate was colorless (*i.e.,* all of the available red phycobiliproteins had been extracted). Chlorophyll and certain other organics then were extracted with 8–10 successive washes of the filtrand, as above, with 50 ml portions of 95% ethanol; these washes were continued until the filtrate was colorless. The remaining filter cake was light tan. The filtrand then was decalcified by uniform dispersion in 100 ml of 5% acetic acid. This slurry was incubated for 1 h with vigorous magnetic stirring, after which it was filtered through a nitrocellulose filter as described above. The resulting decalcified, insoluble crude cell wall preparation was washed extensively, on the filter, with 150 ml of distilled water,

resuspended in 4 ml distilled water (using a Dounce homogenizer), adjusted on a pH meter to pH 8.2 (with about 3 drops of 0.1 *N* NaOH), assayed for morphogenic activity, and stored frozen. The activity of this material remained stable when frozen for at least several weeks.

Treatment with periodate and enzymes; assays for sulfate

Periodate oxidation was performed under standard conditions (Hassid and Abraham, 1957) as a test for the possible dependence of morphogenetic activity on *vic*-glycols typical of carbohydrates. Duplicate 1 ml aliquots of the decalcified cell wall-associated morphogen (see above) were incubated with an equal volume of 0.37 *M* sodium m-periodate for 1 h at 28°C. In one sample, the remaining periodate then was consumed by the addition of a 20-fold molar excess of glycerol; in the control sample, the periodate had been inactivated with this quantity of glycerol *prior* to its addition to the cell wall fraction. Both particulate samples then were washed free of the added chemicals by filtration on nitrocellulose filters (0.2 μm, 25 mm diam.), rinsed with rifampicin-containing filtered seawater, resuspended in this medium, and assayed in duplicate, in their entirety, for morphogenetic activity.

The following purified enzymes were used to analyze the structural determinants of morphogenetic activity, and to solubilize morphogens from the decalcified cell wall fraction. The quantities indicated in parentheses are the amounts used in each separate incubation: sulfatase purified from *Aerobacter aerogenes* (10 units), from *Patella vulgata* (200 units), and from *Haliotis cracherodii* (200 units, except where indicated otherwise in Fig. 5); trypsin (from bovine pancreas, 100 μg); papain (100 μg); β-galactosidase (from *Escherichia coli,* 1000 units); α-galactosidase (from *E. coli,* 10 units); β-glucuronidase (from *Helix aspersa,* 10,000 units); hyaluronidase (from bovine testes, 100 μg); agarase (from *Pseudomonas atlantica,* 1000 units); lysozyme (from chicken egg white, 10,000 units); and endo-β-galactosidase (from *Bacteroides fragilis,* 0.1 unit). Each of these enzymes was of the highest purity commercially available; all were obtained from Sigma (St. Louis), with the exception of endo-β-galactosidase, which was from Boehringer Mannheim (Mannheim). In addition to these highly purified enzymes, a crude preparation of digestive enzymes partially purified from *Haliotis cracherodii* was obtained from Sigma; this mixture, principally containing sulfatase, with lesser amounts of β-glucuronidase and other enzymes, was used in one experiment (Fig. 4) in amounts of 100 μg per incubation.

All enzyme incubations were carried out at 28°C for 1 h in 0.2 μm-filtered seawater containing 2 μg/ml rifampicin, with 0.5–1.0 ml of the final resuspended decalcified, cell wall-associated inducer (see above) used as substrate in each incubation, unless otherwise noted. Incubations of this particulate inducer (substrate) alone, with no added enzymes, were always conducted in parallel with the enzyme digestions.

Two methods were used to separate the enzymes and to solubilize the inducing molecules from the particulate cell wall fraction that was used as substrate. (1) In the first method, the samples were filtered (and washed) on nitrocellulose membrane filters; the remaining particulate cell wall fractions were then resuspended and assayed for retention of morphogenic activity. Assays with the chromogenic substrate, p-nitrophenylsulfate, showed that this method removed all detectable traces of sulfatase from the treated particulate fraction. This method has the further advantage that most of the enzyme proteins were adsorbed to the nitrocellulose filters, so that preliminary assays of soluble morphogen released into the filtrate could be performed (*cf.* Fig. 7). (2) In the second method, designed to ensure complete separation of the *solubilized* morphogens from any possible traces of the enzymes prior to assay, the enzyme digestions were performed inside closed dialysis tubing, and the small soluble morphogens were collected in the external dialysate (20 ml of 0.2 μm-filtered seawater containing 2 μg/ml rifampicin); the morphogens could then be assayed directly, in duplicate 10 ml portions. The use of dialysis membranes with calibrated retention limits of 2,000 Da, 6,000–8,000 Da, and 10,000–14,000 Da (Spectrapor, from Spectrum Medical Industries, Los Angeles) allowed us to estimate the molecular weights of the soluble morphogens thus obtained.

In one additional enzymatic technique, insoluble forms of trypsin and of papain (each covalently conjugated to agarose beads) were used to probe the structural determinants of a soluble, low molecular weight morphogen. This procedure, which affords rapid separation (by filtration) of the morphogen from the enzyme, was necessitated by the instability of the low molecular weight morphogen. The conjugated enzymes were from Sigma.

Sulfate was assayed turbidimetrically by precipitation with barium, after strong acid hydrolysis (Beeley, 1985).

Partial alkaline hydrolysis

The decalcified, cell-wall associated inducer from 100 mg of *Hydrolithon boergesenii* was incubated in 14 ml H_2O, after adjustment with NaOH to pH 12.0, in a sealed tube with vigorous magnetic stirring at 28°C. As a function of time, 1.5 ml aliquots were withdrawn, diluted with a 20-fold excess of 0.2 μm-filtered seawater containing 2 μg/ml rifampicin, and adjusted carefully with HCl to pH 8.2 (±.05). The neutralized samples then were filtered through nitrocellulose filters (0.2 μm, 47 mm diam.) and the soluble fraction assayed (in duplicate 10 ml samples) under the standard conditions.

Molecular weight estimation

The molecular weight of the small morphogen solubilized by partial alkaline hydrolysis was estimated by three independent methods: (1) *Gel-filtration through Sephadex G-10 (exclusion limit = ca. 2000 Da) in a column (0.9 cm diam × 14 cm length) calibrated with the excluded dye, Blue Dextran.* The sample was applied in 2 ml (10 m*M* Tris-Cl, 40 m*M* NaCl, pH 8.2), and eluted in 1 ml fractions of the same buffer. Each fraction was assayed in duplicate 0.5 ml aliquots in a final volume of 10 ml (0.2 μm-filtered seawater containing 2 μg/ml rifampicin) in the standard assay for morphogenic activity.

(2) *Ultrafiltration through Amicon YM-5 calibrated ultrafiltration membranes (retention limit = 5000 Da) in a low-volume Amicon pressure filtration cell.* Duplicate samples of 2 ml (in 0.2 μm-filtered seawater containing 2 μg/ml rifampicin) were filtered, and the resulting filtrates each analyzed in duplicate. Retained molecules were eluted from the upper face of the filters and assayed in duplicate. Molecules adsorbed or trapped on the filters also were assayed directly; finely cut fragments of the entire filters were immersed in the standard assay beakers.

(3) *Dialysis through Spectrapor calibrated dialysis tubing (retention limit = 2000 Da).* Duplicate 2 ml samples (in 0.2 μm-filtered seawater containing 2 μg/ml rifampicin) were dialyzed against 20 ml of the same medium outside the membrane. After the first dialysis for 5 h, the external dialysate was removed, and a second portion of 20 ml was placed outside the membrane for a further 5-h dialysis. At the end of the 10-h period, assays were performed on duplicate 10-ml aliquots of the two external dialysates from each sample and on the sample remaining inside the membrane (duplicate 1-ml aliquots assayed in a final volume of 10 ml). Assays performed on a sample incubated in parallel with no dialysis controlled for loss of activity resulting from inactivation during the 10-h experiment.

Binding to DEAE

Adsorption to the diethylaminoethyl (DEAE)-based anion exchanger was investigated with two different types of DEAE-substituted adsorbant, DEAE-Sephadex (A-50, from Pharmacia, Piscattaway) and DEAE-nitrocellulose filters (NA-45, from Schliecher and Schuell, Keene). For the purpose of these experiments, units of morphogenetic activity were defined as the mean percentage of larvae metamorphosed (in duplicate standard assays after 24 h) per ml of sample (in the linear, concentration-dependent range of the assay below saturation), multiplied by the number of ml in the sample ÷ 1%. When care has been taken to ensure that assays are performed in the linear range, results with the low molecular weight morphogen have proven quantitatively reliable for inventory purposes (*cf.* Table V).

A 3-ml bed-volume column of DEAE-Sephadex A-50 was loaded with 1000 units of the low molecular weight morphogen obtained by mild alkaline hydrolysis. The sample was applied in 18 ml of 1 m*M* Tris-Cl, pH 8.2; the column then was successively eluted with an additional 10 ml of this buffer, followed by 30 ml of the same buffer containing 0.4 *M* NaCl. Fractions of 5 ml were collected, and each was assayed in duplicate portions (of 0.2, 0.5, and 1.0 ml each), in a final volume of 10 ml, for morphogenetic activity. After chromatography, samples of the resin were assayed as well.

DEAE-nitrocellulose filters were pre-washed (with 1 m*M* Tris-Cl, pH 8.2), loaded with 640 units of the low molecular weight morphogen in 4 ml of the same buffer, and then eluted with a syringe. After application of the sample, the filters were washed successively with additional 4 ml portions of: (a) the same buffer; (b) the same buffer containing 0.4 *M* NaCl; and (c) cold 0.4 *N* HCl, at an apparent pH = 0.53. The acid eluate was immediately adjusted to 1 m*M* Tris-Cl and neutralized with NaOH to pH 8.2. Each eluate then was assayed in duplicate 0.5 ml aliquots for morphogenetic activity; the filters were cut into fine segments, rinsed in distilled water, and assayed directly as well.

Results

Persistence of larval competence, stringency and specificity

We had previously found that larvae of *Agaricia humilis* maintain their competence to undergo metamorphosis for at least 12 days following planulation (Morse *et al.*, 1988). The results shown in Figure 1 extend those observations, demonstrating that morphogenetic competence, and the stringency of the larval dependence on an exogenous morphogenetic inducer, are maintained for at least 30 days following larval release. The specificity of the larvae for only certain species of CCA as morphogenetic inducers also persists for this time. Surfaces of the alga *Hydrolithon boergesenii* are recognized as morphogenetic stimuli by larvae 30 days after release, as well as by larvae only hours after release, while surfaces of *Neogoniolithon megacarpum* are not inductive throughout this period (Fig. 1). However, the initial rate of response of the larvae to the *Hydrolithon* inducer declines after about 2 weeks following larval release (Morse and Morse, in prep.). For that reason, all subsequent experiments were performed with larvae within the first 5 days following release.

Morphogen is associated with alga-specific cell wall carbohydrate

The morphogen associated with surfaces of *Hydrolithon boergesenii* was found previously to be detectable in ho-

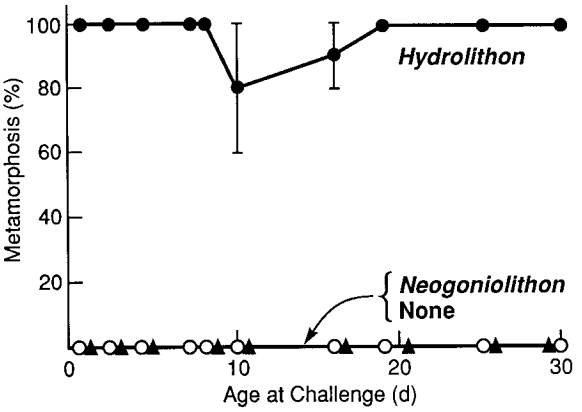

Figure 1. Persistence of stringency and specificity of the requirement of *Agaricia humilis* larvae for specific nongeniculate coralline red algae, *e.g., Hydrolithon boergesenii* (●), to induce settlement and metamorphosis. Larvae exposed to non-inductive *Neogoniolithon megacarpum* (▲) or to no inducer (○) fail to metamorphose. (5 larvae/trial; n = 2–4 trials each; 36 h). Where no error bars are shown, variation = 0.

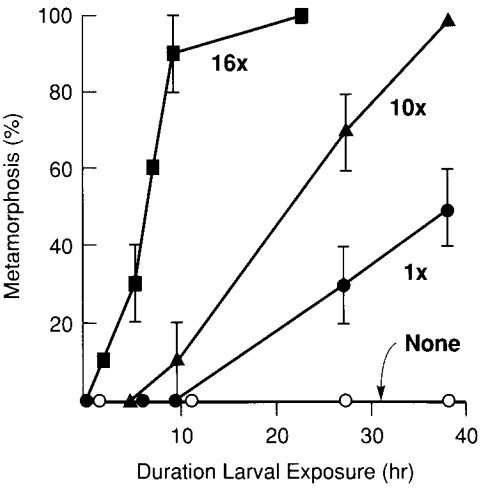

Figure 2. Inducer of larval attachment and metamorphosis is associated with cell wall fraction purified from *Hydrolithon boergesenii* (and associated microsymbionts). Results show time-dependent induction of metamorphosis by 1×, 10×, or 16× relative amounts of cell wall fraction; 1× = 0.02 ml of decalcified cell wall fraction. Standard assay conditions as described in Materials and Methods.

mogenates prepared from the intact alga in seawater, but little or no activity could be found in the soluble filtrates of such homogenates; activity remained associated with the washed, insoluble fraction (Morse *et al.,* 1988). The morphogen was insoluble in water (tested up to 60°C), organic solvents (ethanol, methanol, acetone, and ether), and 5% acetic acid. Preparation of a cell wall fraction from *Hydrolithon boergesenii* (and its associated microsymbionts) by sequential homogenization, extraction of the water-soluble cytoplasmic fraction, removal of chlorophyll and lipids by extraction with ethanol, and decalcification with dilute acetic acid, as described in Materials and Methods, yielded material containing the inducer. The acetic acid decalcification increased the morphogenetic activity of the preparation by about 60–100% relative to that in the seawater-extracted crude homogenate (data not shown). The resulting crude cell wall preparation shows strong morphogenetic activity that is both dose- and time-dependent (Fig. 2), with lower amounts of the material exhibiting a pronounced lag in the induction of larval metamorphosis. Two species of nongeniculate red algae (*Porolithon pachydermum* and *Neogoniolithon megacarpum*) that do not induce *A. humilis* larvae to settle or metamorphose lack the chemical inducer associated with the cell wall fraction (Table I). Thus the recognition of this chemical inducer is likely to be the basis for the observed algal substratum specificity.

The decalcified, cell wall-associated morphogen from *Hydrolithon boergesenii* was tested for its sensitivity to inactivation by exposure to the mild chemical oxidant, sodium-*m*-periodate. Exposure to periodate for 1 h at 28°C, followed by inactivation of the remaining periodate with glycerol, and washing of the treated sample by ultrafiltration, completely inactivated the morphogen (Fig. 3). Activity also could not be detected in the filtrate of the periodate-treated sample. As seen in Figure 3, a control sample treated in parallel, but receiving "periodate" only after the oxidant had first been inactivated with glycerol, retained full morphogenetic activity. The observed sensitivity of the morphogen, and the known specificity of the oxidant under the conditions used (Hassid and Abraham, 1957), suggest that the morphogen may contain a carbohydrate or other *vic*-glycol moiety essential for its activity.

Enzyme probes reveal essential features of morphogen structure

The association of the morphogen with a decalcified crude cell wall preparation (Fig. 2) and its sensitivity to

Table I

Non-inductive algae lack the cell wall-associated chemical inducer

	Metamorphosis (%)		
Addition	*Hydrolithon boergesenii*	*Porolithon pachydermum*	*Neogoniolithon megacarpum*
Alga	100 ± 0	0 ± 0	0 ± 0
Homogenate	60 ± 0	0 ± 0	0 ± 0
Cell wall fraction	100 ± 0	0 ± 0	0 ± 0

Larvae of *Agaricia humilis* were assayed for metamorphic response to three species of nongeniculate coralline red algae, and in parallel, to the crude homogenates and decalcified cell wall fractions prepared from these algae. Metamorphosis scored at 48 h; other details as in Figure 1.

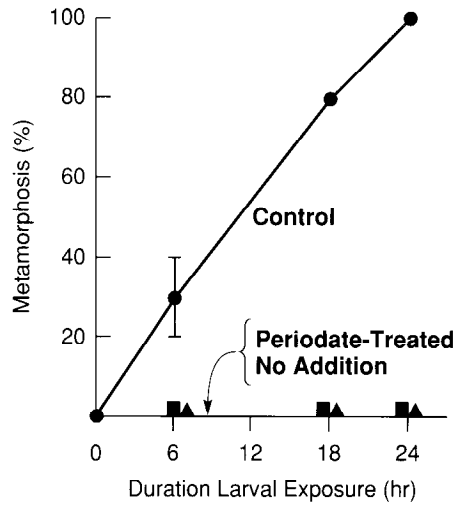

Figure 3. Inactivation of the cell wall-associated inducer of larval metamorphosis by oxidation with periodate. The control and periodate-treated samples of cell wall-associated inducer (equal amounts, incubated in parallel) differed only in the time at which the periodate was inactivated with glycerol. In the control sample, the periodate was inactivated prior to its addition to the inducer; in the treated sample, the periodate was inactivated 1 h after exposure to the inducer. Further details are in Materials and Methods. All assays in duplicate.

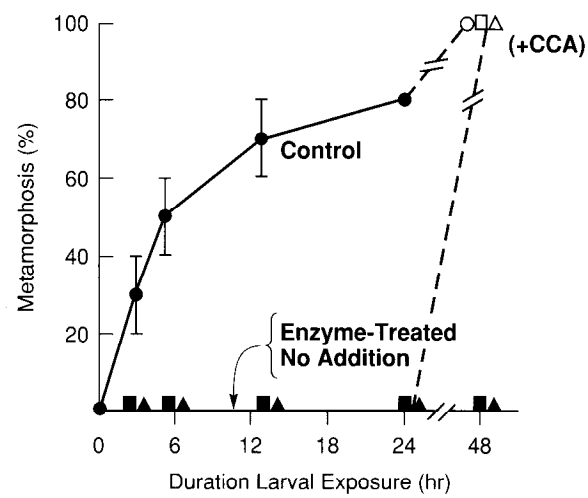

Figure 4. Inactivation of the cell wall-associated inducer by treatment with digestive enzymes from abalone. Equivalent amounts of inducer were incubated in parallel (1 h, 28°C) with or without exposure to enzymes. Samples then were washed by ultrafiltration. Assays with the chromogenic substrate for sulfatase, p-nitrophenylsulfate, confirmed that all of the enzyme was removed from the particulate samples by this washing procedure. The washed samples then were assayed in quadruplicate for remaining morphogenic activity (in parallel with assays with no addition). After 24 h of exposure of the larvae to each of the three assay conditions, fresh samples of the inductive crustose coralline red alga *Hydrolithon boergesenii* (CCA) were added to duplicate samples of each assay type (open symbols), to assess the remaining responsiveness of the larvae.

periodate (Fig. 3) strongly suggest the presence of a carbohydrate, and possibly a polysaccharide, component. This suggestion has been confirmed, and essential features of the morphogenic polysaccharide have been revealed, through the use of enzymes as structural probes.

The morphogenic activity of the cell wall preparation proved sensitive to a crude mixture of sulfatase and other digestive enzymes from the gut of the herbivorous gastropod, *Haliotis cracheroddi* (black abalone). Equivalent amounts of the inducer were incubated in the presence and absence of the abalone digestive enzymes for 1 h at 28°C; after this incubation, the enzymes were removed and the cell wall fractions washed by ultrafiltration. Equivalent portions then were assayed, in quadruplicate, with *Agaricia humilis* larvae (Fig. 4). This enzyme treatment completely inactivated the cell wall-associated morphogen. Assays of the filtrate (not shown) confirmed that the active moiety had been destroyed, and not simply solubilized. In the experiment illustrated in Figure 4, small samples of untreated *Hydrolithon boergesenii* were added to duplicate groups of larvae that had been incubated for 24 h in the presence of the treated or untreated inducer, or incubated for that period in the absence of inducer. All of these larvae (100% ± 0%) metamorphosed in response to the added coralline alga, indicating that the prior enzyme treatment had in no way affected the larvae; *i.e.*, the enzyme had inactivated the morphogen, and not the larvae.

Similar results were obtained when the morphogen was treated with the digestive sulfatase purified from the limpet, *Patella vulgata* (Table II). A significant reduction in the dose-dependent activity of the cell wall-associated morphogen resulted from this enzyme treatment. When

Table II

Effect of sulfatase purified from Patella vulgata *on cell wall-associated morphogen*

Inducer (equivalents)[1]		Metamorphosis[2] (%)
Untreated	Enzyme-treated	
0	0	0 ± 0
1	0	70 ± 10
6	0	100 ± 0
0	1	0 ± 0
0	6	20 ± 0
1	1	70 ± 10

[1] The decalcified morphogen was incubated with or without enzyme for 1 h at 28°C, and subsequently washed free of enzyme, as described in Materials and Methods. One equivalent corresponds to 0.25 ml of the original decalcified morphogen. Portions of the two samples then were assayed, either singly or in pre-mixed combination, in the relative amounts shown.

[2] Assays were conducted with 5 larvae/trial, and scored for metamorphosis after 24 h; results = mean ± S.D.; n = 2 trials for each condition.

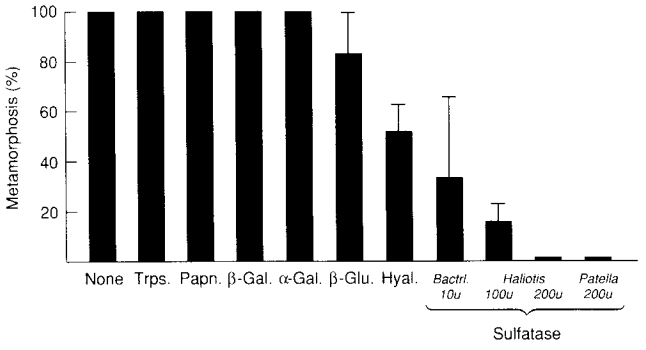

Figure 5. Effects of purified enzymes used as probes for structural determinants of the cell wall-associated inducer of larval metamorphosis. Trps. = trypsin; Papn. = papain; β-Gal. = β-galactosidase; α-Gal. = α-galactosidase; β-Glu. = β-glucuronidase; Hyal. = hyaluronidase; sulfatases purified from the bacterium *Aerobacter aerogenes,* the limpet *Patella vulgata,* and the abalone *Haliotis cracherodii,* were used in the amounts (enzyme units) indicated. Assays, after treatment and washing of the particulate samples, were performed in duplicate; all other details as in Figure 4 and Materials and Methods.

equivalent amounts of the enzyme-treated and the untreated morphogen were mixed and assayed together, no reduction in the activity of the untreated morphogen was observed, thus confirming the conclusion that the molluscan enzymes inactivated the inducer, and not the larvae. Larvae exposed to molluscan digestive enzyme-treated inducer, and either subsequently (Fig. 4) or simultaneously (Table II) exposed to untreated inducer, responded normally and completed metamorphosis.

The digestive enzyme preparation from *Haliotis* that inactivated the coral morphogen (Fig. 4) is a relatively crude mixture containing high quantities of a sulfatase (Spaulding and Morse, 1991) and lower quantities of β-glucuronidase and several other enzymes. When purified molluscan sulfatase and β-glucuronidase were tested separately, the sulfatase was a potent inactivator of the cell wall-associated morphogen, whereas β-glucuronidase had little if any significant activity (Fig. 5). The data show that sulfatases purified from *Haliotis, Patella,* and from a bacterium all inactivate the morphogen, and that the effect of the *Haliotis* sulfatase is concentration-dependent. (The slight inactivation caused by treatment with the β-glucuronidase preparation is likely to reflect the activity of the small amount of sulfatase known to still contaminate this preparation.)

In similar tests, five other purified enzymes were used to probe for essential features of the morphogen structure. Of these, only hyaluronidase, which cleaves sulfated polysaccharide chains, reduced the activity of the insoluble, cell wall-associated morphogen (Fig. 5). The proteolytic enzymes trypsin and papain, and the exosaccharidases α-galactosidase and β-galactosidase, were completely without effect. These results, and those presented above, strongly suggest that a sulfated polysaccharide is an essential component of the morphogen recognized by the *Agaricia humilis* larvae.

Enzymes release and degrade soluble morphogen

Four purified endopolysaccharidases, including agarase, endo-β-galactosidase, lysozyme, and hyaluronidase, released a soluble morphogen from the insoluble cell wall preparation (Fig. 6). The subsequent time-dependent decline in soluble morphogen activity seen with prolonged exposure to agarase suggests that this enzyme may continue to attack the solubilized inducer; this suggestion is confirmed by experiments presented below. None of the other enzymes tested in the experiment shown in Figure 5 released any detectable soluble activity.

The enzyme-solubilized morphogen was a potent inducer, causing the *Agaricia humilis* larvae to quickly attach either to the sides or bottoms of the clean polystyrene assay beakers, and to undergo rapid and normal metamorphosis and post-metamorphic growth (*cf.* Fig. 10). These results, and those with the alkali-solubilized material discussed below, prove that the larvae do not require any specific tactile stimulus from the morphogen, but respond solely to its chemical structure.

Experiments in which aliquots of the cell wall-associated morphogen were incubated in the presence and absence of purified enzymes, inside dialysis tubing, confirmed that agarase and other endosaccharidases continue to attack and subsequently degrade the soluble morphogenic molecules they first release (as suggested by the data in Fig.

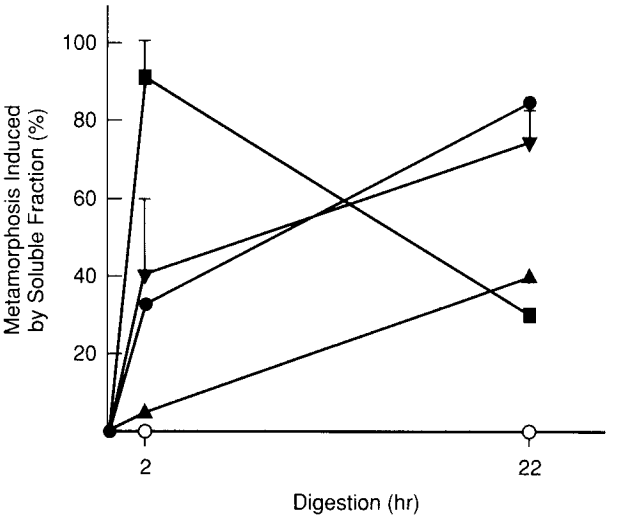

Figure 6. Solubilization of morphogen by treatment with purified endopolysaccharidases. Agarase (squares); endo-β-galactosidase (filled circles); hyaluronidase (upward triangles); lysozyme (downward triangles); no enzyme controls (open circles). Other procedures similar to experiment shown in Figure 5. Details are in Materials and Methods.

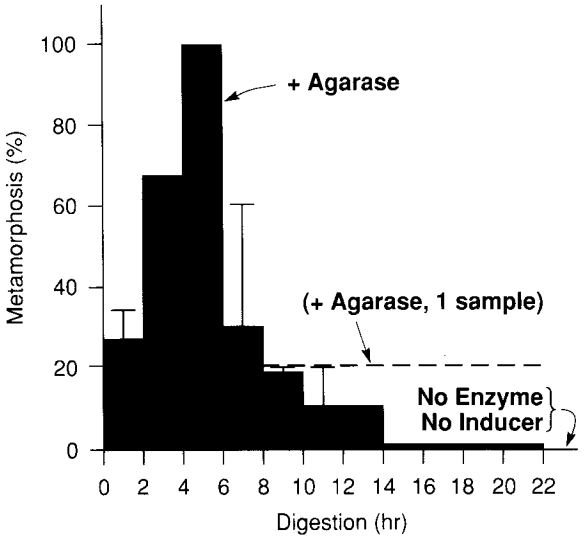

Figure 7. Enzymatic solubilization and further purification of small morphogen. The insoluble, cell wall-associated inducer of larval metamorphosis was digested inside a dialysis tubing (retention limit = 14,000 Da) with purified agarase. Small morphogens released from the insoluble inducer were allowed to accumulate in the external dialysate, which was removed and assayed, and replaced with fresh external medium, every two hours. An otherwise identical sample was digested in parallel with no change of dialysate for 22 h, after which the external medium was removed and assayed (dotted line). Other details as described in the text; all assays were performed in duplicate under standard conditions. Controls conducted in parallel included assays of dialysates changed every 2 h from a sample incubated with no enzyme, and larvae incubated with no additions; these gave 0 ± 0% metamorphosis. Results show metamorphosis induced by the dialysates.

6). The external dialysates (including the permeant small morphogens released) were allowed to diffuse and accumulate outside of the membranes for a fixed interval, after which they were removed for assay, and replaced with fresh external liquid for the next interval of incubation. With purified agarase, and 2-h intervals for accumulation, removal, and replacement of the external dialysates, we observed a time-dependent increase and subsequent decline in the rate of appearance of dialyzable morphogenic activity with $\bar{M} \leq 14{,}000$ Da (Fig. 7). However, far less of the total morphogenic activity could be detected in an otherwise identical incubation, in which only one sample of dialysate was collected and assayed after prolonged incubation (24 h). All of the dialysates were held at 28°C until the end of the experiment (24 h) and assayed simultaneously, to control for any time-dependent decay of activity. Because the difference between the two parallel incubations thus was in the limitation (2 h interval) or prolongation (24 h interval) of the opportunity for the small dialyzable morphogen to diffuse back and forth between the outside and inside of the dialysis tubing, we conclude that prolonged exposure of released, dialyzable morphogen to the agarase resulted in degradation of the active morphogen. We therefore conclude that agarase, which cleaves sulfated polysaccharides, particularly at β-1,4-linked galactose residues, not only releases the active morphogen from a sulfated polysaccharide parent molecule, but degrades the morphogen itself, as well. Therefore, the structure of the morphogen would seem to contain a sulfated polysaccharide with galactose residues.

In similar experiments, we compared dialysis membranes with calibrated porosites of *ca.* 10,000–14,000 Da and *ca.* 6,000–8,000 Da, and two other endosaccharidases in addition to agarase (Fig. 8). When lysozyme was used we again saw a time-dependent increase and subsequent decline in the rate of accumulation of dialyzable morphogen with $\bar{M} \leq 14{,}000$ Da. There was a similar, but displaced, rise and fall in the accumulation of smaller morphogen ($\bar{M} \leq 8000$ Da), suggesting that these smaller molecules may be derived, in part, by enzymatic cleavage of the larger dialyzable inducers. With the 8-h intervals

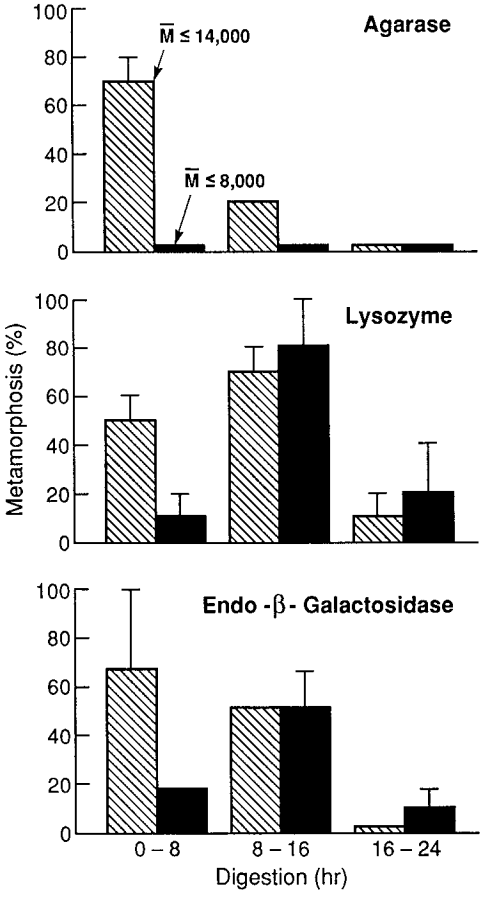

Figure 8. Enzymatic solubilization and further purification of small morphogen through dialysis membranes of two different porosities. Experiment performed as in Figure 7, except that: agarase, lysozyme and endo-β-galactosidase were compared; the digestions were performed in parallel in dialysis tubings with retention limits of 14,000 Da (hatched bars) and 8,000 Da (solid bars); the external dialysates were changed and saved for assays every 8 h.

Table III

Specificities and effects of enzyme probes used to solubilize and characterize the morphogen

Enzyme	Cleaves specifically[2]	Effect of morphogen[3]			
		Inactivates	Solubilizes	$\bar{M} \leq 14{,}000$	$\bar{M} \leq 8{,}000$
Proteases					
Papain	Peptides	No	No	—	—
Trypsin	Peptides at Lys, Arg	No	No	—	—
Exosaccharidases					
β-Glucuronidase	Terminal β-glucuronate glycosides	No	No	—	—
β-Galactosidase	Terminal β-galactosides	No	No	—	—
α-Galactosidase	Terminal α-galactosides	No	No	—	—
Sulfatases[1]					
Sulfatase-1	Organic sulfate esters	Yes	No	—	—
Sulfatase-2	Organic sulfate esters	Yes	No	—	—
Sulfatase-3	Organic sulfate esters	Yes	No	—	—
Endosaccharidases					
Hyaluronidase	Sulfated polysac. chains	(Yes)	(Yes)	No	No
Agarase	Galactans & sulfated galactan chains at β-1,4 linkages	(Yes)	Yes	Yes	No
Lysozyme	Polysac. chains at β-1,4 linkages to N-acetyl-glucosamine	(Yes)	Yes	Yes	Yes
Endo-β-galactosidase	Polysac. chains at internal β-1,4, linkages between galactose and N-acetyl-glucosamine or N-acetyl-glucosamine-sulfate	(Yes)	Yes	Yes	Yes

[1] Sulfatases -1, -2, and -3 = enzymes purified from *Haliotis, Patella,* and *Aerobacter,* respectively.

[2] Enzyme specificities: endo-β-galactosidase (Li *et al.,* 1982; Kitamikado *et al.,* 1982; Scudder *et al.,* 1983, 1984); β-agarase (Morrice *et al.,,* 1983a, b; Usov and Ivanova, 1987); sulfatase (De *et al.,* 1978; Dodgson and Fitzgerald, 1982); others (Zubay, 1983; Freifelder, 1983).

[3] Solubilization refers to the release of active morphogen into a 0.2 μm ultrafiltrate; M values refer to release of small dialyzable morphogen with the indicated approximate molecular weight. Parentheses indicate that the effect is observed but slow.

for dialysate accumulation employed in the experiment shown in Figure 8 (compared to the 2-h intervals employed in Fig. 7), only a time-dependent decline in the rate of accumulation of morphogen ≤ 14,000 Da was observed when digestion was catalyzed by endo-β-galactosidase. A slower, time-dependent rise and subsequent fall in accumulation of smaller morphogenic molecules ($\bar{M} \leq 8{,}000$ Da) is seen, however, again suggesting that some of these smaller molecules may be produced by cleavage of the larger but still dialyzable morphogens. The activity of the small dialyzable morphogen released by endo-β-galactosidase was unaffected by incubation for several hours in the presence or absence of trypsin or papain linked to insoluble matrices (data not shown), confirming the earlier finding (*cf.* Fig. 5) that the morphogen is resistant to these proteases.

With agarase catalyzing cleavage, the 8-h collection intervals permit only the observation of the time-dependent decline in the rate of accumulation of morphogen ≤ 14,000 Da (Fig. 8). Little or no active morphogen ≤ 8,000 Da was observed from the agarase digestion, suggesting perhaps that the smaller fragments produced by this enzyme either lack essential morphogenetic determinants, or may be particularly labile to subsequent inactivation (either spontaneous or enzymatic). Similar experiments with hyaluronidase produced no detectable morphogen in the dialysates from either porosity membrane (data not shown). This failure of hyaluronidase to yield dialyzable morphogen might reflect either the failure of cleavages catalyzed by this enzyme to yield molecules sufficiently small to leave the dialysis tubing, or the destruction by hyaluronidase of sites required for morphogenic activity.

The substrate specificities of the enzyme probes employed in these studies, and the results observed, are summarized in Table III. Reduction in the yield of the low molecular weight ($\bar{M} \leq 8{,}000$ Da) morphogen when any two of the tested endopolysaccharidases are present (results not shown) suggests that this small molecule itself contains sites cleaved by agarase, lysozyme, and hyaluronidase (as does the cell wall-associated complex from which the morphogen can be released by these enzymes). A similar conclusion had been drawn independently from the experiments with agarase alone (Figs. 6, 7). The substrate specificities of these purified enzymes (Table III) thus suggest that the small morphogen contains a sulfated

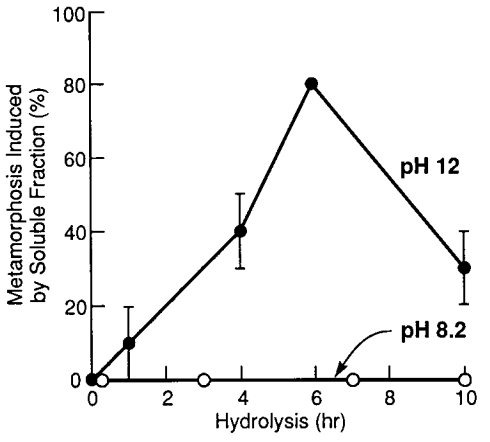

Figure 9. Time-course of solubilization of morphogen by mild alkaline hydrolysis. The decalcified, cell wall-associated inducer from *Hydrolithon boergesenii* was incubated with vigorous stirring at pH 12.0. At the times indicated, aliquots were withdrawn, neutralized to pH 8.2, filtered through nitrocellulose filters (0.2 μm, 47 mm diam.), and the soluble fractions assayed (in duplicate 10 ml samples) under the standard conditions. Details are in Materials and Methods. Results shown are the average percentages of larvae induced to attach and metamorphose by the soluble fractions, ±S.D. Results of a parallel and otherwise identical incubation held continuously at pH 8.2 are included for comparison.

polysaccharide (cleaved by hyaluronidase, agarase, and sulfatase), with multiple, substituted residues of N-acetylglucosamine (linkages cleaved by lysozyme and endo-β-galactosidase) and multiple residues of galactose (linkages cleaved by agarase and endo-β-galactosidase). Protein (or peptide) does not appear to be an essential component, nor do several specific free or terminal sugars or saccharide units.

Hydrolysis with dilute alkali releases small anionic morphogen containing sulfate

The finding that specific endopolysaccharidases can release a soluble morphogen from the insoluble cell wall-associated fraction suggested that non-enzymatic, partial hydrolysis under mild conditions might yield similar results. While some desulfation also would occur, hydrolysis with sufficiently dilute alkali would be expected to yield oligosaccharide fragments with retention of at least some of the original sulfate groups (Percival and McDowell, 1967). As predicted, exposure of the decalcified, particulate cell wall fraction to dilute alkali (pH 11–12) released a soluble, sulfate-containing morphogen. The increase in yield of soluble morphogenic activity as a function of the time of incubation at pH 12 is shown in Figure 9. The apparent decline in yield after 6 h suggests that the morphogen itself is slowly inactivated at pH 12. Exposure to strong acid (4 *N* HCl) or strong base (4 *N* NaOH) causes more rapid inactivation.

The morphogen released by mild alkaline hydrolysis is of relatively low molecular weight, strongly anionic, sulfate-containing, and unstable. Estimates of the apparent molecular weight were made by three independent methods: ultrafiltration through a calibrated membrane, dialysis through a calibrated dialysis membrane, and Sephadex gel filtration (Table IV). In ultrafiltration, all of the applied activity was recovered in the YM-5 membrane ultrafiltrates, consistent with $\bar{M} \leq 5000$ Da. In dialysis through a calibrated membrane, all of the recovered activity (equal to that in the non-dialyzed control) was found in the first external dialysate, (see Materials and Methods), consistent with $\bar{M} \leq 2000$ Da. These results were confirmed by gel-filtration through Sephadex G-10, in which all of the applied activity was eluted at the end of the included volume, indicating $\bar{M} \leq 2000$ Da. These findings all indicate, therefore, that the morphogen solubilized by alkaline hydrolysis may be as small as 2000 Da or less (Table IV).

This low molecular weight material is strongly anionic; it binds tightly to DEAE Sephadex and to DEAE-nitrocellulose (Table V). Because the material does not bind to Sephadex or nitrocellulose alone, without DEAE substitution, the binding is most likely dependent on ionic interaction. This binding proves to be very strong; little activity could be removed from the DEAE Sephadex by elution with 0.4 *M* NaCl, and 75% of the initially applied activity was found still adsorbed to the resin (Table V). Similarly, 63% ± 13% of the activity applied to a DEAE-nitrocellulose filter was found still adsorbed to the filter, even after elution with 0.4 *N* HCl. That the morphogen was not eluted from DEAE by 0.4 *N* HCl indicates that its ionic binding was dependent on more strongly anionic groups than uronic acids or other simple carboxylates (pK *ca.* 2). These results are consistent with the conclusion, based on sulfatase sensitivity (*cf.* Figs. 4, 5; Table II), that the morphogen contains sulfate groups (see Discussion).

The presence of sulfate esters in this solubilized morphogen was confirmed independently by turbidimetric analysis with barium after strong acid hydrolysis (method according to Beeley, 1985). The yields obtained indicate

Table IV

Estimation of molecular weight of the small morphogen solubilized by partial alkaline hydrolysis

Method	$\bar{M}$ Estimate (Da)
Ultrafiltration	≤5000
Dialysis	≤2000
Sephadex gel filtration	≤2000

Details are as described in Materials and Methods. Independent experiments verified that there was no significant adsorption of the morphogen to the gel-filtration matrix or to the ultrafiltration or dialysis membranes.

Table V

Adsorption of the small morphogen, solubilized by partial alkaline hydrolysis, to DEAE

Adsorbant	Eluant or fraction	Units	Recovery (%)
DEAE Sephadex	Application	1,000	—
	1 m*M* Tris	0	0
	1 m*M* Tris + 0.4 *M* NaCl	50	5
	Remaining on DEAE	753	75.3
	(Total)		(80.3)
DEAE Nitrocellulose	Application	640	—
	1 m*M* Tris	0	0
	1 m*M* Tris + 0.4 *M* NaCl	0	0
	0.4 *N* HCl	0	0
	Remaining on DEAE	400 ± 80	63 ± 13
	(Total)		(63 ± 13)

Details are as described in Materials and Methods. Morphogen recovered in the high-salt eluate, and that remaining on the DEAE, induced larvae to attach firmly to the polystyrene assay beakers and metamorphose normally. Control experiments demonstrated that the DEAE adsorbants without the applied morphogen had no such activity. Additional controls showed that there is no binding of the soluble morphogen either to Sephadex or nitrocellulose alone, without the DEAE ion-exchange groups present.

the presence of *ca.* 8–14% (w/w) sulfate in the alkali-solubilized morphogenic fraction.

Agaricia humilis larvae exposed to the alkali-solubilized low molecular weight morphogen, either free in solution or bound to DEAE (Sephadex or nitrocellulose) showed rapid, normal, and complete metamorphosis. The activity of both the soluble and DEAE-bound morphogen was concentration- or dose-dependent.

Activity, specificity, and stability of the small soluble morphogen

The small, dialyzable morphogens obtained in the experiments shown in Figures 6–9 induce rapid and normal larval settlement, attachment, complete metamorphosis, and normal post-metamorphic growth (Fig. 10). These processes induced by the small morphogenic molecules are indistinguishable from those induced by the native, intact nongeniculate coralline alga.

The low molecular weight morphogens produced by enzymatic or alkaline hydrolysis of the particulate cell wall fraction from *Hydrolithon boergesenii* (or associated microbial symbionts) induce metamorphosis of at least one other agariciid coral, in addition to *Agaricia humilis.* Larvae of the sympatric *A. tenuifolia,* which also are induced to settle and metamorphose by the intact *Hydrolithon boergesenii* (A. Morse *et al.,* in prep.), are induced to metamorphose by the dialyzable morphogen released by agarase digestion of the cell wall fraction from this alga, with an efficiency comparable to that exhibited for the *A. humilis* larvae (Table VI). In both of these species, the coral larvae are induced to attach to the walls or bottom of the polystyrene containers, metamorphose completely, and begin normal post-metamorphic growth in response only to the low molecular weight chemical morphogen. In contrast, larvae of the sympatric ahermatypic coral, *Tubastraea aurea,* which are not induced to metamorphose by *Hydrolithon boergesenii,* are not induced by the low molecular weight morphogen enzymatically released from the algal cell wall fraction (Table VI). This demonstrates that the morphogenic activity of this chemical is biologically specific for those larvae that respond to the intact alga.

Whereas the decalcified, cell wall-associated, insoluble morphogen is relatively stable when frozen at −10°C, the small, dialyzable morphogen ($\bar{M} \leq 8000$ Da) released from this parent material by endo-β-galactosidase is stable for only a few days at −10°C. The lower molecular weight morphogen released by mild alkaline hydrolysis ($\bar{M} \leq 2000$) proved to be even more unstable. Even when frozen at −10°C, this material lost activity with a half-life that varied in different preparations between 24 and 72 h.

Activities of known compounds

We have tested a wide variety of sulfated and non-sulfated polysaccharides and related polymers, including several substrates for the enzymes used in this study. These (all pre-adjusted to the pH of ambient seawater prior to testing) have included: agar; agarose; agaric acid; alginic acid; ascophyllan; λ-, ι-, and κ-carrageenans; fucoidan, furcellaran; laminarin; chitin; di-N-acetylchitobiose; tri-N-acetylchitotriose; keratan sulfate; chondroitin sulfates A, B, and C; heparin; heparan sulfate; hyaluronic acid; asialofetuin; dextran sulfate; pentosan sulfate; polyvinyl sulfate; polyanethol sulfate; pectin; amylopectin; cellulose; cellulose sulfate; sulfoethylcellulose; and sulfopropyl-sepharose. Most of these proved inactive with *Agaricia humilis* larvae. Only the κ-carrageenan (a sulfated polymer rich in galactose), fucoidan (a sulfated polymer rich in fucose), and keratan sulfate (a sulfated glycosaminoglycan) induced metamorphosis, although this activity was weak and evident only at very high concentrations (≥5 mg/ml).

Discussion

Recent field and laboratory studies have shown that recruitment of the shallow-water agariciid corals, *Agaricia humilis* and *A. tenuifolia,* is determined in part by larval recognition of a chemical inducer of substratum-specific settlement and metamorphosis (Morse *et al.,* 1988). This inducer is associated with specific nongeniculate coralline red algae. In the case of *A. humilis,* only certain coralline

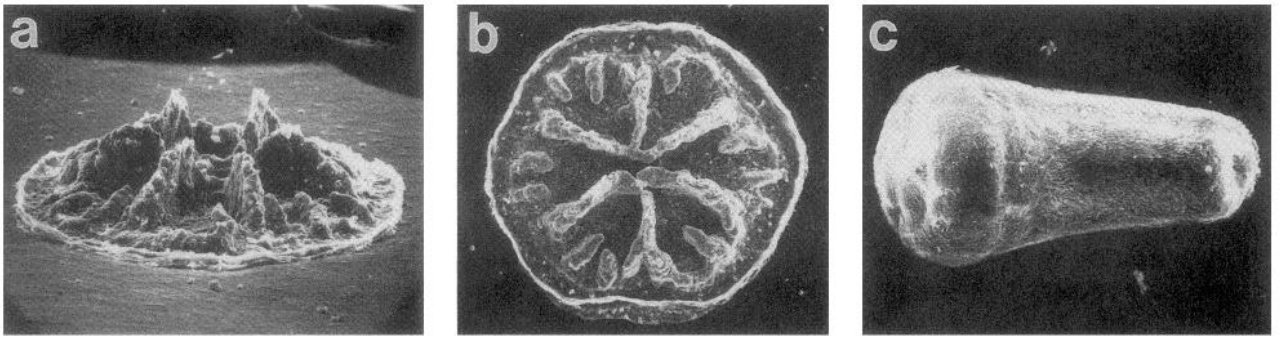

Figure 10. Normal attachment and metamorphosis of *Agaricia humilis* larva induced by the soluble morphogen. Scanning electron micrographs of the endoskeleton of a post-metamorphic corallite attached to polystyrene, dorsolateral (a) and dorsal (b) views, and for comparison, a pre-metamorphic planula larva (c). The pronounced calcified septa and the attachment plaque are clearly visible in the 2-day post-metamorphic corallite. Diameter of the corallite *ca.* 1.5 ± 0.1 mm; length of the planula *ca.* 0.7 ± 0.1 mm. The soluble morphogen was obtained by digestion with endo-β-galactosidase, followed by dialysis, as in Figure 8. Tissue-digestion, fixation, dehydration, critical-point drying, and electron microscopy were by standard procedures.

red algae contain the inducing morphogen (Fig. 1; Table I; *cf.* Morse *et al.*, 1988; A. Morse and R. Steneck, in prep.).

We have extended our previous finding that *Agaricia humilis* larvae maintain both the stringency of their dependence upon the alga-associated morphogen, and the specificity of this requirement, for at least 30 days following their release (Fig. 1). While most larvae probably settle and metamorphose in less than 30 days in the natural environment, this capacity to delay metamorphosis in the absence of the required morphogen can enhance both the dispersal of the larvae, and the substratum-specificity of the final distribution of recruits. Although much of scleractinian recruitment may be locally seeded (Bak and Engle, 1979; Rylaarsdam, 1983; Baggett and Bright, 1985; Sammarco and Andrews, 1988), the larvae of *A. humilis* (*cf.* Fig. 1; also Morse *et al.*, 1988) and certain other species (*cf.* Harrigan, 1972; Richmond, 1981, 1985, 1987; Harrison *et al.*, 1984; Scheltema, 1986; Morse *et al.*, 1988; Richmond and Hunter, 1990) are capable of distant dispersal as well. Indeed, temporal pulses of scleractinian recruitment at certain sites on the Great Barrier Reef are dependent on the settlement of larvae produced at distant locations (Wallace, 1985; Babcock, 1988). In addition to the importance of hydrodynamic, topographic, and geographic features for the control of larval dispersal and consequent recruitment (*e.g.*, Frith *et al.*, 1986; Roughgarden *et al.*, 1988; Sammarco and Andrews, 1988; Black, 1988), and the effects of predation, nutrition, competition, and other biotic factors, studies of *A. humilis* (Morse *et al.*, 1988; and those reported here) indicate that for some coral species, the stringency and specificity of the larval requirements for the induction of settlement and metamorphosis also can be important in controlling the spatial distribution of recruits. A similar larval requirement for a substratum-specific biochemical inducer of settlement

Table VI

Induction of metamorphosis of Agaricia humilis *and* A. tenuifolia *larvae, and absence of induction of* Tubastraea aurea, *by the low molecular weight (LMW) morphogen*

Treatment	Metamorphosis at 24 h ($\bar{X}$ ± S.D.)		
	Agaricia humilis	*Agaricia tenuifolia*	*Tubastraea aurea*
LMW morphogen	95% ± 10% (n = 4)	87% ± 23% (n = 3)	0% ± 0% (n = 3)
Seawater control	0% ± 0% (n = 5)	0% ± 0% (n = 5)	0% ± 0% (n = 3)

Soluble morphogen was prepared by agarase digestion of the particulate cell wall preparation of *Hydrolithon boergesenii* (agarase, 1 mg, 1000 units), and dialysis through a membrane retaining molecules with $\bar{M} \geq 14{,}000$ Da, as described in Materials and Methods. Larvae of each of the three coral species were assayed for metamorphosis in response to the dialyzable morphogen and seawater controls in parallel; 5 larvae/trial; n = number of trials.

and metamorphosis, and larval recognition of the chemical inducer in the ocean environment, recently were shown to determine the fine-scale spatial distribution of recruitment of the polychaete, *Phragmatopoma californica* (Jensen and Morse, 1990).

Chemical nature of the inducer

The inducer of *Agaricia humilis* settlement and metamorphosis that is associated with specific nongeniculate coralline red algae is chemical in nature (Table I; *cf.* Morse *et al.,* 1988). This morphogenic substance is insoluble in a wide range of solvents, apparently because it is associated with cell wall polysaccharides (with which the morphogen is partially purified; *cf.* Fig. 2). Solubilized fractions of the inducer can be generated, however, by hydrolysis with enzymes or mild alkali. The fact that these solubilized fractions are sufficient to trigger normal attachment and metamorphosis of *A. humilis* larvae on clean polystyrene surfaces (Fig. 10) proves that the requirement of the larvae for a morphogenetic inducer is satisfied by a chemical substance from the inductive algal substratum. Non-recruiting algal substrata do not yield this activity (Table I). These results thus confirm and extend the finding that larval recognition of the inductive but insoluble particulate fraction, partially purified from the settlement-inducing algal surface, is dependent upon the integrity of a periodate-sensitive (Fig. 3) and sulfatase-sensitive (Fig. 5; Table II) chemical structure. These sensitivities, the insolubility of the crude inducer, and its insensitivity to proteolytic enzymes, suggested that the inducer is associated with a sulfated polysaccharide.

Consistent with this suggestion, we have found that the inducer can be solubilized by cleavage with purified endoglycosidases that can act on sulfated polysaccharides (Figs. 6–8). As summarized in Table III, the cleavage by agarase and by endo-β-galactosidase indicates that the substrate polymer contains galactose units; cleavage by lysozyme and by endo-β-galactosidase indicates that this polymer also contains β-1,4 linked N-acetylglucosamine or N-acetylglucosamine sulfate units (*cf.* Li *et al.,* 1982; Kitamikado *et al.,* 1982; Scudder *et al.,* 1983, 1984; Morrice *et al.,* 1983a, b; Usov and Ivanova, 1987). Because continued hydrolysis by each of these enzymes leads first to solubilization, and then to progressive inactivation of the inducer, the inductive moiety itself probably contains the above-mentioned sites of hydrolysis. The morphogenetic inducer is thus associated with, and may itself contain, a sulfated glycosaminoglycan, *i.e.,* a sulfated polysaccharide that includes multiple N-acetylglucosamine and galactose units. The finding that partial alkaline hydrolysis liberates a sulfate-containing, strongly anionic, small morphogen is consistent with this conclusion. Recently, hydrolysis, ion-exchange HPLC, and sensitive detection of the resolved monosaccharides by pulsed amperometry have independently confirmed that glucosamine (derived from N-acetylglucosamine) and galactose are indeed principal components of this solubilized small morphogen (M. Hardy, Dionex Corp., pers. comm.). Sulfated polysaccharides that contain amino sugars and are rich in galactose, similar to those in foliose red algae (*cf.,* Percival and McDowell, 1967; Percival, 1978; McCandless, 1981; Yaphe, 1984), previously were found in other coralline red algae (Turvey and Simpson, 1965).

Keratan sulfate, which proved to be slightly active as a morphogen, has three characteristics that make it unique among the known sulfated glycosaminoglycans tested. In these same features, it also, to some degree, resembles the morphogen recognized by *Agaricia humilis* larvae: (1) it contains multiple galactose-β-1,4-N-acetylglucosamine units (whereas heparin, heparan sulfate, and the three different chondroitin sulfates all contain glucuronic acid or other uronic acid units in place of galactose); (2) it is readily cleaved by endo-β-galactosidase (Scudder *et al.,* 1983), whereas the other known compounds tested are not; and (3) the keratan sulfates from some sources cannot be eluted from anion exchangers at salt concentrations below 3–4 *M* (Rodén *et al.,* 1972; *cf.* our results with DEAE, Table V), whereas the other sulfated glycosaminoglycans are readily eluted at significantly lower concentrations (Rodén *et al.,* 1972). Very highly sulfated galactans from red algae also are not eluted from DEAE, or else are eluted only at high concentrations of urea or at high temperature (Yaphe, 1984). However, the weak morphogenetic activity evident at only high concentrations of keratan sulfate, carrageenan, and fucoidan indicates that although these substances may be somewhat similar to the natural inducer, they differ in structural features (possibly including positions of the sulfate groups or other linkages) from those of the natural morphogen.

The morphogenetic activity of the natural inducer solubilized from the cell wall fraction obtained from *Hydrolithon boergesenii* or its associated microflora is biologically specific (Table VI). This molecule induces normal attachment and metamorphosis in larvae of two species of *Agaricia* that are also induced by the intact alga from which the morphogen was obtained. However, the molecule fails to induce these reactions in larvae of the sympatric *Tubastraea aurea,* which are not induced by the intact alga.

The high specificity of the *Agaricia humilis* larvae for morphogens associated with only certain nongeniculate coralline red algae (Fig. 1; Table I; Morse *et al.,* 1988; A. Morse and R. Steneck, in prep.) is apparently a reflection of the chemical specificity of the larval receptors for only some unique sulfated glycosaminoglycans. This suggestion is supported by the finding that a wide variety of synthetic natural sulfated polysaccharides, glycosaminoglycans, and

structurally related polymers have either little or no morphogenetic activity. A better understanding of the chemical basis for the high specificity of the larval receptors awaits further information about the stereochemistry and structure of the organic sulfate esters and other substituents in the natural morphogen.

Relation to other systems

Sulfated glycosaminoglycans and other sulfated polysaccharides have been widely implicated in other highly specific cell recognition phenomena that control differentiation, including inter-phyletic symbiosis, fertilization, aggregation of sponge cells, the "homing" of circulating mammalian lymphocytes into the lymph nodes, pattern formation in the developing nervous system, and the metastasis and invasiveness of tumors. The number of structural permutations of such molecules, and hence the complexity of coding of cell recognition ideotypes, far exceed those of the proteins, which are more fully understood (Drickamer, 1988; Sharon and Lis, 1989).

Recent evidence has shown that the host specificity for nodule induction in leguminous plants by the nitrogen-fixing symbiotic bacterium, *Rhizobium meliloti,* is determined by recognition of a unique sulfated glycosaminoglycan signal (a tetrasaccharide containing one sulfate and one acylamino group) produced by the bacterium (Lerouge *et al.,* 1990). Recognition, by the host plant, of this inducing bacterial signal, and the resulting specificity of the inter-phyletic symbiosis, has long been thought to be governed by a class of receptors known as lectins, present on the plant root hair surfaces (Kijne *et al.,* 1989). Recently, this suggestion was strongly confirmed when the intergeneric transfer between plants of the DNA coding for one such root lectin resulted in the transfer of host specificity for nodulation induced by the bacterium (Diaz *et al.,* 1989). Recognition of specific sulfated polysaccharides at the surface of *Strongylocentrotus purpuratus* sea urchin eggs by conspecific sperm has been demonstrated to be essential for fertilization (Rossignol *et al.,* 1984; DeAngelis and Glabe, 1987). In this reaction (DeAngelis and Glabe, 1987), and in the binding of heparin to antithrombin (Atha *et al.,* 1985), the locations of the sulfate esters on the sugars have been found to be of critical importance; in the latter case, removal of one specific sulfate reduced the affinity of specific binding by as much as 10,000-fold (Atha *et al.,* 1985). The lengths of the sulfated polysaccharides are also critical determinants of the strength (and hence, the specificity) of these binding reactions (Hoylaerts *et al.,* 1984; DeAngelis and Glabe, 1987). Evidence also suggests that lectin-like recognition of cell surface sulfated glycosaminoglycans controls an essential phase in the species-specific reaggregation and subsequent differentiation of sponge cells (Henkart *et al.,* 1973; Turner and Burger, 1973; Jumblatt *et al.,* 1980; Conrad *et al.,* 1984; Diehl-Seifert *et al.,* 1985, 1989; Margoliash *et al.,* 1965; Coombe *et al.,* 1987; Coombe and Parish, 1988; Schröder *et al.,* 1988; Gramzow *et al.,* 1989).

Recognition and binding of sulfated glycosaminoglycan moieties of cell surface proteoglycans also controls adhesion, tissue-specific differentiation and growth in a wide variety of mammalian and other higher systems (Edelman, 1985; Fransson, 1987; Ruoslahti, 1989). The structures and positions of the sulfate esters can be critically important in the control of these functions as well (Fransson, 1987). Sulfated glycosaminoglycans and other sulfated polysaccharides bind to lectin-like receptors on the surfaces of lymphocytes (Parish *et al.,* 1984; Parish and Snowden, 1985; Chong and Parish, 1985, 1986; Thurn and Underhill, 1986; Brenan and Parish, 1986; Brandley *et al.,* 1987). Recently, the genes coding for two distinct "homing receptors" from the surfaces of mammalian lymphocytes have been cloned and sequenced (Gallatin *et al.,* 1986; Yednock *et al.,* 1987a; Goldstein *et al.,* 1989; Holzmann *et al.,* 1989; Siegelman *et al.,* 1989; Stamenkovic *et al.,* 1989; Stoolman, 1989). Lectin-like recognition of specific sulfated or other anionic carbohydrates in the target lymphoid tissues, mediated by these receptors, is thought to direct the homing of specific subsets of lymphocytes from the circulation to adhere to the blood vessel endothelia of their target lymphoid organs (lymph nodes, Peyer's patches, etc.), where the recruited lymphocytes then differentiate to produce antibodies (Brenan and Parish, 1986; Gallatin *et al.,* 1986; Yednock *et al.,* 1987a, b; Jalkanen *et al.,* 1988; Stoolman, 1989; Coombe and Rider, 1989).

The parallels between this lymphocyte homing reaction and the settlement and metamorphosis of *Agaricia humilis* larvae are potentially interesting. In both, substratum-specific "recruitment," attachment, and differentiation are apparently induced by recognition of a non-diffusing, substratum-specific sulfated polysaccharide. Our further observation that the partially purified sulfated polysaccharide that induces *A. humilis* larvae to attach and metamorphose also induces murine lymphocytes to undergo mitosis (Morse and Eardley, unpub. obs.), may therefore be worth further investigation. The specificity of this latter reaction is unclear, however, as a wide variety of sulfated polysaccharides and related polymers, including several carrageenans, fucoidan, and ascophylan, induce lymphocyte mitosis. Whether the same subset of lymphocytes responds to each of these compounds has not yet been determined. In contrast, the *A. humilis* larvae respond only slightly and incompletely to carrageenan and fucoidan, and only at very high concentrations.

Cell-surface recognition of polysaccharides and other complex carbohydrates in such non-immune systems is generally considered to be mediated by lectins, a broad

class of ubiquitous, carbohydrate-specific receptors that recently has been redefined (Barondes, 1988; Drickamer, 1988; Sharon and Lis, 1989). Mitchell and his colleagues first demonstrated that the settlement and metamorphosis of larvae of the polychaete, *Janua brasiliensis,* are mediated by a lectin-like recognition of inductive exopolysaccharides produced by specific bacteria (Kirchman *et al.,* 1982a, b; Mitchell and Kirchman, 1984; Maki and Mitchell, 1985, 1986). These authors first pointed out the similarities between this larval settlement reaction and other lectin-mediated recognition phenomena, including the root nodule-bacteria symbiosis discussed above. Weiner *et al.* (1985) and Bonar *et al.* (1986) also have shown that specific bacterial exopolysaccharides may play a role in the induction of settlement and metamorphosis of *Crassostrea virginica* and *C. gigas* oyster larvae, although induction in those systems is complex, and more than one class of compound is known to be involved (Coon *et al.,* 1985; Coon and Bonar, 1987; Fitt *et al.,* 1989; Bonar *et al.,* 1990).

The results reported here, demonstrating sensitivity of the *Agaricia humilis* morphogen to cleavage by endo-β-galactosidase and agarase, (and resistance to the exoglycosidic β-galactosidase), indicate that the morphogen contains essential internal β-galactoside units. This finding may be of particular interest in view of the suggested importance of β-galactoside-specific lectins in controlling differentiation in higher animal systems (Barondes *et al.,* 1988; Sharon and Lis, 1989).

Remaining problems

Two problems remaining are the determination of the complete chemical structure of the inducing molecule recognized by the *Agaricia humilis* larvae, and the unequivocal identification of the biological source of this inducer. A number of compounds that induce metamorphosis of various marine invertebrate larvae have been partially purified from the respective inductive substrata, and the structures of these compounds have been partially characterized. But we are aware of only two natural inductive molecules that have been completely characterized. These include the algal molecules that induce metamorphosis of the scallop, *Pecten maximus* (Yvin *et al.,* 1985), and the hydrozoan, *Coryne uchidai* (Kato *et al.,* 1975). Significantly, both of these are small molecules soluble in organic solvents, and thus amenable to gas-chromatography and mass spectroscopy. In a large number of the other cases investigated, however, the native inducers have proved to be either water soluble (*e.g.,* Highsmith, 1982; Hadfield and Scheuer, 1985; Burke, 1986; Hadfield and Pennington, 1990), or polymeric and insoluble (*e.g.,* Jensen and Morse, 1984, 1990; Morse *et al.,* 1988). [We are not including in this discussion such molecules as potassium or calcium salts, fatty acids, cyclic nucleotides, or other widely active effectors of depolarization, protein phosphorylation, or signal transduction pathways; these all have been shown to induce metamorphosis of larvae without species- or substratum-specificity (*e.g.,* Baloun and Morse, 1984; Morse, 1985, 1990; Yool *et al.,* 1986; Pechenik and Heyman, 1987; Jensen and Morse, 1990; Jensen *et al.,* 1990)]. The inducer of *Agaricia humilis* metamorphosis described here is in its native form associated with a substratum-specific insoluble polymer. Partial hydrolysis with either enzymes or dilute alkali releases a smaller, water-soluble and strongly anionic inducer which is markedly unstable. This instability has hindered analyses of the active morphogen. The use of highly purified enzymes, and employment of their specificities for selective cleavage, solubilization, and as probes of the structural determinants of morphogenetic activity, may prove widely useful in further studies of such otherwise intractable molecules.

The inductive molecule that we have described is obtained from homogenates of the nongeniculate coralline red alga, *Hydrolithon boergesenii.* Larvae of *Agaricia humilis* are induced to metamorphose by contact with intact specimens of that alga, and recruits of the coral are found preferentially on that alga in the field (Morse *et al.,* 1988; A. Morse and R. Steneck, in prep.). Three other species of anthozoan, including the scleractinian *Agaricia tenuifolia* (Morse *et al.,* 1988), the temperate octocoral *Alcyonium siderium* (Sebens, 1983a, b), and a tropical gorgonian, *Plexaura* sp. (Lasker, 1990), also have been found to settle and metamorphose in response to crustose red algal surfaces. But in each of these cases, the inductive molecule could have been produced by bacteria or other microorganisms associated with the algal surfaces. Bacteria or bacterial films have been implicated in the control of larval settlement and metamorphosis in a few other cnidarians in which these processes are chemically induced. Larvae of the hydroid, *Hydractinia echinata,* settle and metamorphose in response to films of the bacterium, *Alteromonas* sp., on shells inhabited by hermit crabs (Spindler and Müller, 1972). *Cassiopea andromeda* (scyphozoan) larvae also are induced to settle and metamorphose by bacteria, apparently in response to soluble peptides produced by the action of bacterial degradative enzymes (Fitt and Hofmann, 1985; Fitt *et al.,* 1987; Hofmann and Brand, 1987). Bacterial films have been widely implicated in the control of larval settlement and metamorphosis in many other kinds of invertebrates as well (Wilson, 1955; Cameron and Hinegardner, 1974; Brancato and Woolacott, 1982; Kirchman *et al.,* 1982; Mitchell and Kirchman, 1984; Bonar *et al.,* 1986; Maki and Mitchell, 1986; Fitt *et al.,* 1989; Maki *et al.,* 1990). Moreover, as Maki *et al.* (1990) have suggested in the case of barnacle larvae, the larval response may depend in a complex way on the

interaction between bacteria and the surface on which they are attached.

The structure of the morphogen recognized by *Agaricia humilis* larvae, suggested by the results reported here to be a sulfated glycosaminoglycan, would be equally consistent with a molecule of the red algal cell wall and of a cell wall or other exopolymer produced by an associated bacterium (Percival and McDowell, 1967; Mackie and Preston, 1974; Sanford *et al.*, 1977; McCandless, 1981; Drews and Weckesser, 1982; Boyle and Reed, 1983). We have found, however, that the activity of crude homogenates appears markedly enhanced following decalcification, consistent with the unmasking of constituents of the algal cell wall. Attempts to culture the algal cells axenically from isolated protoplasts, by the methods of Polne-Fuller and Gibor (1984) and Kloareg *et al.* (1989), and attempts to culture the alga-associated microbial symbionts, may help further resolve the source of the inducer. Mass-cultivation of the producing cells would also help provide sufficient quantities of the inducer and thus facilitate its complete structural characterization.

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