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INHIBITION AND INDUCTION OF BARNACLE SETTLEMENT BY NATURAL PRODUCTS PRESENT IN OCTOCORALS

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Abstract—Barnacle settlement inhibitors and inducers are present in the gorgonian *Leptogorgia virgulata* and the pennatulacean *Renilla reniformis*. The inhibitors are low-molecular-weight compounds (<20,000 daltons) that were detected in soft tissue homogenates and dialysates of homogenate and in ambient "gorgonian water." Settlement was almost completely inhibited at a dialysate concentration of 1.0 g wet weight equivalents/liter. The inhibitors probably function in chemical defense against predation and fouling, and could prove useful in ship fouling control. The settlement inducers are high-molecular-weight substances (>20,000 daltons) that adsorb to surfaces.

Key Words—Inhibition, induction, chemical defense, larval settlement, fouling, barnacle, *Balanus amphitrite amphitrite*, octocorals, *Leptogorgia virgulata, Renilla reniformis.*

INTRODUCTION

Chemical interactions between organisms are relatively well known in terrestrial communities (Sondheimer and Simeone, 1970; Whittaker and Feeny, 1971). It is now clear that marine communities are also organized around a variety of chemical messages affecting the behavior and distribution of organisms (Kittredge et al., 1974). Particularly important to community development, and to the fouling of surfaces, is the chemical information involved in larval settlement (Crisp, 1974, 1976).

Larval settlement can be affected by two major kinds of allelochemics:

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inducers and inhibitors. Inducers are substances that encourage settlement and metamorphosis. Barnacle cyprids, for example, settle gregariously near barnacle spat and adults (Knight-Jones and Stephenson, 1950; Knight-Jones, 1953), and this response is probably due to cyprid contact with adsorbed proteins (Crisp and Meadows, 1962, 1963; Larman and Gabbott, 1975). Inducers are also implicated when the larvae of predators or symbionts settle specifically on their prey or host organisms (Scheltema, 1974; Lewis, 1978). Only a few studies have succeeded in identifying settlement inducers (Kato et al., 1975; Morse et al., 1979).

Allelochemics that discourage settlement and metamorphosis—settlement inhibitors—have received less study. Barnacle settlement may be suppressed by specific microbes or microbial films (D'Agostino and Sheridan, 1969) and by the tannins present in certain brown algae (Sieburth and Conover, 1965), but no data are given in these reports. In fact, settlement inhibition by natural products is poorly understood in any animal, and convincing evidence has yet to be reported in barnacles, probably because it is difficult to show a settlement difference between an inhibition treatment that approaches zero and a chemically neutral control that may not be much higher.

We report here that settlement of the barnacle *Balanus amphitrite amphitrite* Darwin, 1854 is inhibited by substances present in the gorgonian *Leptogorgia virgulata* (Lamarck, 1815) and the pennatulacean *Renilla reniformis* (Pallas, 1766). Curiously, these same octocorals have inducers that promote barnacle settlement.

METHODS AND MATERIALS

Octocoral Preparation. Colonies of *Leptogorgia virgulata* were collected by free-diving on the jetty at Radio Island, near Beaufort, North Carolina. *Renilla reniformis* were obtained by trawling in the adjacent Newport River estuary. The animals were maintained in the laboratory in running seawater for no more than two days before being used. Most of the work was accomplished in the summer and fall.

Soft tissues of the octocorals were weighed and homogenized in full strength ($34^0/_{00}$) seawater with a tissue grinder, followed by centrifugation (12,000 *g*) and fiber filtration (Whatman No. 1). The resulting "homogenate" was diluted with seawater to concentrations of 1.0 or 2.0 g soft tissue wet weight equivalents/liter and used as test water. In most of the experiments, however, the homogenate was dialyzed for 24 hr at 4° C in 100 times its volume of stirred seawater. This treatment separated the homogenate into dialysate and retentate fractions, both of which were used in tests after further dilution. In two experiments most of the bacteria were removed from undialyzed

gorgonian homogenate. This procedure was accomplished in three test treatments by the addition of antibiotics (15 mg sodium penicillin and 25 mg streptomycin sulfate/liter), by ultrafiltration (0.22-μm membrane filter), and by ultraviolet irradiation (close exposure to UV lamp for 14 min). In another experiment, "gorgonian water" was tested instead of homogenate or dialysate. This water was obtained by immersing healthy, living *Leptogorgia* in gently aerated seawater ($34^0/_{00}$) for 18 hr at 15° C. The water surrounding the colonies was then fiber-filtered and used in tests at 17.0 and 3.4 g soft tissue wet weight equivalents/liter.

Barnacle Rearing. Cyprid larvae of the intertidal barnacle *Balanus amphitrite amphitrite* were the assay organisms. These were obtained by rearing first stage nauplius larvae to the cyprid stage in the laboratory. The nauplii were obtained either by dissecting field-collected adults for ripe embryo masses or by collecting naturally spawned nauplii from adults maintained in breeding condition in the laboratory. These latter adults were fed on diatoms (*Skeletonema costatum*) and brine shrimp (*Artemia salina*) nauplii. All of the adult barnacles were collected from Pivers Island, near Beaufort.

Once obtained, the barnacle nauplii were reared in gently aerated polypropylene carboys at densities of about 800 nauplii/liter of glass fiber-filtered seawater ($34^0/_{00}$). The carboys were kept in environmental chambers at a constant temperature of 25° C and a 15-hr light:9-hr dark photoperiod. Nauplii were fed daily on *Skeletonema costatum* at a density of 30,000 cells/ml. The diatom was cultured separately in autoclaved seawater enriched with f/2 nutrients (Guillard, 1975). Under these conditions, first-stage nauplii were reared to cyprids in about five days, and survival was about 40%. Cyprids were then filtered from the cultures and kept in beakers of clean seawater at 15° C for one day before testing. The beakers were coated with a thin film of paraffin to minimize settlement on the glass.

Experimental Design. For each experiment cyprids were mixed by magnetic stirring, and approximately equal numbers were aliquotted into 200-ml polystyrene drinking cups that served as replicate test chambers. The cyprids in each cup were then filtered from the aliquot water and rinsed back into the cup with test water or control seawater to a final volume of 200 ml. A single settling substrate was then added to the bottom of each cup. For most of the experiments, the substrates were slate rectangles ($0.4 \times 2 \times 5$ cm) bearing ten drilled pits on their upper surfaces to promote settlement. These were identical to those used by Crisp and Meadows (1963) and were cleaned in the same way they suggest before being reused. In several experiments screened hardboard paneling was used for settlement substrates. These substrates were the same size as the slates but were soaked in running tap water for 24 hr before the tests to make them negatively buoyant. Cyprids settled almost

entirely in the tiny rough gouges covering the upper surfaces. The hardboard substrates elicited higher settlement in controls than the slates and were disposable.

Two experiments designed to test adsorption of gorgonian retentate to slate substrates involved some different procedures. In these experiments slates were first soaked in seawater or retentate without cyprids for 3 h. They were then dipped in seawater three times to remove most of the unadsorbed organic materials and finally placed in cups containing either seawater or retentate for testing with cyprids.

The cups in any single experiment were placed around the periphery of a large turntable with the long axes of the substrates arranged circumferentially. The turntable rotated at 0.7 rpm, serving to minimize lighting differences. Cups were arranged in replicate groups around the circumference; each group contained all of the experimental treatments, and treatments were assigned randomly within each group. The apparatus was located in an environmental chamber having constant temperature (25° C) and light intensity (7.0×10^{15} quanta/cm^2/sec).

Experiments ran for 12–48 hr, depending on settlement rate. When settlement ended, the replicates were preserved in 70% alcohol. Permanently attached cyprids and spat on the upper surface of each substrate were then counted and summed to give the total number of settled barnacles. The number of unattached cyprids was also counted, permitting the calculation of mean percentage settlement.

Experiments were analyzed statistically with single-classification ANOVAs (F). Percentage settlement data underwent an angular transformation prior to testing. The Student-Newman-Keuls test was used to compare treatment means.

RESULTS

Leptogorgia *Inhibitor.* In three experiments gorgonian homogenates elicited significantly lower settlement than seawater controls ($P < 0.001$; Table 1). For example, experiment 1 had eight replicates and an average of 322 cyprids available for settlement in each replicate. In this experiment nearly 31% of control cyprids settled, while only 6.5% of those exposed to homogenate settled. Homogenate concentrations of about 10^{-1} g wet weight equivalents/liter appeared to inhibit settlement.

It seemed possible, however, that the active material was produced by associated microbes rather than by the gorgonians. In two experiments (Table 2) settlement was significantly higher in seawater controls than in both unaltered homogenates and homogenates treated with antibiotics, ultrafiltration, and UV irradiation ($F = 5.1$, $0.01 < P < 0.025$ experiment 1;

TABLE 1. EFFECTS OF *Leptogorgia virgulata* HOMOGENATES, DIALYSATES, AND RETENTATES ON BARNACLE SETTLEMENT

Experiments	Mean percentage settlement				Number of replicates	Mean number of cyprids available for settlement	Concentrations (g wet weight) equivalents/liter)
	Seawater controls	Homogenates	Dialysates	Retentates			
1	30.9	6.5			8	322	1.0
2	26.5	1.0	0		4	108	2.0
3	14.7		5.0		10	229	1.0
4	10.5	2.3	0	45.1	5	381	1.0

TABLE 2. EFFECTS OF "LOW-BACTERIA" HOMOGENATES OF *Leptogorgia* ON BARNACLE SETTLEMENT

Experiments	Seawater controls	Unaltered homogenates	"Low-bacteria" homogenates			Number of replicates	Concentrations of homogenate (g wet weight equivalents/liter)
			Antibiotics	Ultrafiltration	Ultraviolet irradiation		
1	4.6[a]	1.6	1.4	2.3		8	2.0
2	128 (30.9)[b]	25 (6.5)	22 (6.4)	40 (10.9)	25 (7.1)	4	1.0

[a]Numbers not enclosed in parentheses indicate mean numbers of settled barnacles.
[b]Numbers in parentheses refer to mean percentage settlement.

$F = 65.7$, $P < 0.001$ in experiment 2). None of the homogenate treatments were significantly different from each other, except that the ultrafiltrate treatment was different from the others in experiment 2. The settlement inhibition effect was apparent whether or not bacteria were present in appreciable numbers.

To begin isolation of the active inhibitor from crude homogenate extracts, we dialyzed these extracts. This technique separates complex, high-molecular-weight retentate molecules from small dialysate molecules, with separation at about 20,000 daltons. Table 1 shows an inhibition effect in the low-molecular-weight dialysate fraction. Dialysate treatments had significantly lower settlement than seawater controls ($P < 0.001$), as was the case with the homogenates. However, dialysates were more active than undialyzed homogenates, sometimes inhibiting all settlement at 1.0 g wet weight equivalents/liter.

Other work on *Leptogorgia* dialysates has concerned concentration levels that inhibit settlement (Figure 1). In four experiments, settlement was inversely proportional to concentration. The two lowest concentrations were not significantly different from controls, but all other concentrations were

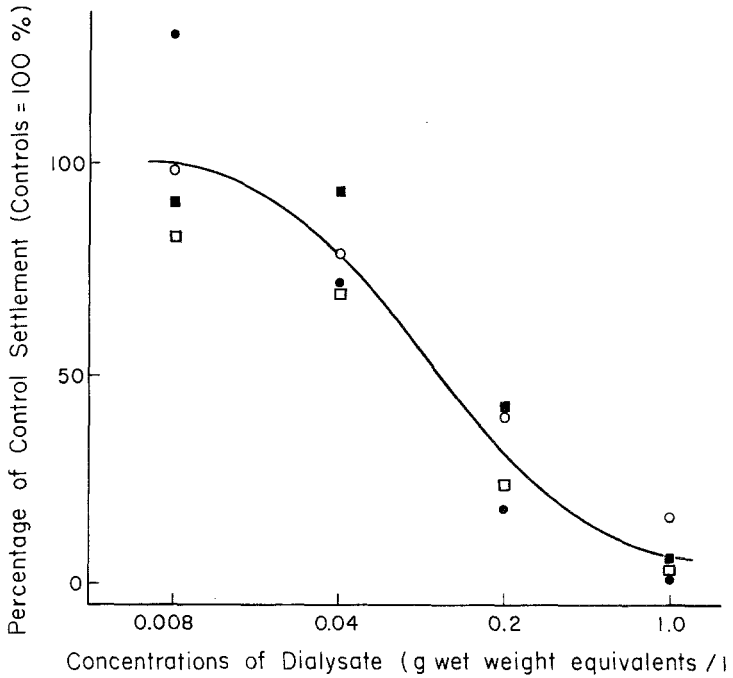

FIG. 1. Effects of concentration of *Leptogorgia* dialysate on barnacle settlement. Dose-response curve represents mean of four experiments, each with four concentrations; each point indicates mean of four replicates.

significantly different from each other ($F = 14.8$; $P < 0.001$). Settlement was almost completely inhibited at 10^{-1} g wet weight equivalents/liter. Cyprids exposed to dialysate appeared less active than those in control seawater but were not killed at the concentrations used. High concentrations of inhibitor also suppressed metamorphosis from attached cyprid to spat.

We have also shown a settlement inhibition effect in "*Leptogorgia* water" obtained in the laboratory. Seawater controls were compared with "gorgonian water" concentrations of 3.4 and 17.0 g wet weight equivalents/liter. Mean settlement percentages in these three treatments were 65.2, 50.3, and 12.6, respectively, and all of them were significantly different from each other ($F = 51.7$; $P < 0.001$). Each treatment consisted of six replicates, and the mean number of cyprids available for settlement in each replicate was 284. As expected, the inhibitor present in the "gorgonian water" was not as concentrated as that present in dialysate or homogenate.

Leptogorgia *Inducer.* In the retentate treatment of experiment 4 (Table 1) settlement was 34.6% higher than in seawater controls, and the difference was highly significant ($F = 169.5$; $P < 0.001$). These data suggest the existence of a high-molecular-weight settlement inducer in the retentate. Thus, the soft tissue of *Leptogorgia* has both a low-molecular-weight inhibitor and a high-molecular-weight inducer. The inhibition effect predominates in undialyzed homogenate, but when the homogenate is dialyzed, induction is more easily demonstrated than inhibition. Both effects were demonstrated repeatedly in experiments designed mainly for other purposes (e.g., Figure 1 and Table 3).

Important to experimental methodology, perception mechanisms, and biofouling control is the extent to which these natural products are active when adsorbed to surfaces. In two experiments involving all combinations of substrate soaking and testing in seawater and retentate (Table 3), the control treatment that was both soaked and tested in seawater had significantly lower settlement than the three retentate treatments ($F = 6.7$, $0.001 < P < 0.005$ in experiment 1; $F = 14.7$, $P < 0.001$ in experiment 2). The retentate treatments were not significantly different from each other. In experiment 4 (Table 1) only 36% of the cyprids in the retentate treatment settled in the pits, which are physically favorable for settlement; the remaining cyprids (64%) settled on the less-favorable smooth surfaces between the pits, presumably because the inducer had adsorbed onto all surfaces and was a more powerful stimulus than the pit contours. These experiments suggest that the inducer adsorbs to slate substrates and is not easily rinsed off. Similar experiments with the inhibitor did not provide clear results.

Renilla *Inhibitor and Inducer.* In addition to the *Leptogorgia* work, we have discovered a second inhibitor and another inducer in the pennatulacean octocoral *Renilla reniformis* (Table 4). As with the gorgonian, the settlement inhibition effect is present in both the undialyzed homogenate and the

TABLE 3. ADSORPTION OF *Leptogorgia* RETENTATES ON SLATE SETTLEMENT SUBSTRATES

	Slates: soaked/tested				
	Controls[a]	Retentate treatments[a,b]			Mean number of cyprids available for settlement
Experiments	Seawater/seawater	Seawater/retentate	Retentate/seawater	Retentate/retentate	
1	0.3[c]	18.5	14.4	9.4	175
2	9.2	25.8	38.5	35.4	302

[a] Each treatment consisted of three replicates.
[b] Concentrations were all 1.0 g wet weight equivalents/liter.
[c] Numbers refer to mean percentage barnacle settlement.

TABLE 4. EFFECTS OF *Renilla reniformis* HOMOGENATES, DIALYSATES, AND RETENTATES ON BARNACLE SETTLEMENT

	Mean percentage settlement				Number of replicates	Mean number of cyprids available for settlement	Concentrations (g wet weight equivalents/liter)
Experiments	Seawater controls	Homogenates	Dialysates	Retentates			
1	30.9	0			4	370	1.0
2	15.1	2.5	0.2	61.7	5	200	1.0

dialysate, with a slightly greater (although not significantly different) effect in the dialysate than in the homogenate. All other differences were significant ($F = 402.5$, $P < 0.001$ in experiment 1; $F = 68.9$, $P < 0.001$ in experiment 2). *Renilla* is also similar to *Leptogorgia* in that a settlement inducer was present in the retentate fraction (Table 4, experiment 2).

DISCUSSION

Our work began with the observation that healthy colonies of *Leptogorgia virgulata* were remarkably free of free-living barnacles and other fouling organisms. Although planktonic cyprids of at least eight free-living barnacle species occur in the immediate vicinity of the gorgonians, the adults of only two of these were to be found on the colonies, and then only rarely. In contrast, dead gorgonians became heavily fouled in a few weeks (Burkholder, 1973; J.D.S., personal observations). Settlement inhibition seemed a likely explanation for this apparent anomaly.

Inhibitors. The present work demonstrates that barnacle settlement inhibitors are present in the soft tissue of *Leptogorgia virgulata* and *Renilla reniformis* (Tables 1 and 4, Figure 1). The inhibitors are low-molecular-weight molecules that can be collected from the water surrounding gorgonians, at least in the laboratory. They effectively inhibit settlement at low concentrations (10^{-2} to 10^{-1} wet weight equivalents/liter; Figure 1) by suppressing cyprid activity, but higher concentrations could be quite toxic. In nature they probably function to chemically defend the octocorals against predation and fouling.

Although no previous studies have dealt with *L. virgulata* and *R. reniformis,* there is a substantial literature on octocoral chemistry. Much of this work concerns the terpenoids that are so ubiquitous in these animals (Ciereszko and Karns, 1973; Fenical, 1978; Tursch et al., 1978). Like our inhibitors, these low-molecular-weight compounds are toxic in low concentrations (Burkholder and Burkholder, 1958; Perkins and Ciereszko, 1973). For example, a cembranolide terpenoid that is a potent neuromuscular toxin occurs in *Lophogorgia* spp. (Fenical et al., 1981), a genus closely related to *Leptogorgia.* Particularly relevant to the octocorals may be the antimicrobial and icthyotoxic effects that have been studied (Burkholder, 1973; Bakus, 1974; Tursch, 1976). Octocoral defense against larval settlement was first proposed by Ciereszko (1962), and Hadfield and Ciereszko (1978) showed that several cembranolides isolated from gorgonians were very toxic to larvae of the nudibranch mollusc *Phestilla sibogae.* No previous studies have focused on larval barnacles, but D.J. Faulkner found juvenile barnacles to be highly susceptible to crassin acetate from *Pseudoplexaura* spp. (footnoted in Wein-

heimer and Matson, 1975). Terpenoids would seem to be likely candidates for the settlement inhibitors described herein.

The present work suggests the possible importance of natural settlement inhibitors to biofouling control. Fouling is controlled nowadays with paints containing inorganic copper or organic tin compounds, or with halogens. Unfortunately, these toxins may pose long-term pollution hazards in inland waters (Hoare and Hiscock, 1974; Good et al., 1979). Natural products, however, may be more biodegradable than heavy metals, thus restricting their toxicity both spatially and temporally. Further evaluation of their use as antifoulants would require isolation studies, toxicity analyses, and development of binding and release technologies.

Inducers. Work on the inhibitors in *Leptogorgia* and *Renilla* led us to the discovery of settlement inducers in the soft tissue retentate fractions of these same octocorals (Tables 1, 3, and 4). Unlike the inhibitors, the inducers are high-molecular-weight substances that adsorb to surfaces. In these respects they appear similar to the adsorbed proteins responsible for the gregarious settlement of barnacles (Crisp and Meadows, 1962, 1963; Larman and Gabbott, 1975). Although *Leptogorgia* colonies are generally free of free-living barnacles and other fouling organisms, they do harbor several symbiotic animals (Patton, 1972). One of these is the interesting barnacle *Conopea galeata* (=*Balanus galeatus*), which occurs exclusively on gorgonians. Settlement of this barnacle could be dependent on gorgonian inducers.

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