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# Large molecules and chemical control of feeding behavior in the starfish *Asterias forbesi*

M. A. Heeb

*Department of Marine Bio-Medical Research, University of North Carolina at Wilmington; Wilmington, N.C., USA*

KURZFASSUNG: **Makromoleküle und chemische Kontrolle des Freßverhaltens bei dem Seestern *Asterias forbesi*.** *Asterias forbesi* reagiert auf gewisse chemische Reizstoffe in einer Weise, die stark an das normale Freßverhalten erinnert; insbesondere kann die sogenannte „Humping"-Reaktion als Grundlage für eine quantitative Auswertung des Reizstoffgehaltes geeigneter Gewebsextrakte benutzt werden. Es wird nachgewiesen, daß Extrakte der Muscheln *Crassostrea virginica* und *Mercenaria mercenaria* diesen Reflex auslösen können und daß die aktiven Bestandteile zum großen Teil in den Fraktionen konzentriert sind, die Moleküle relativ hohen Molekulargewichtes enthalten. Die Aktivität ist hitzebeständig, fällt unter Einwirkung von $(NH_4)_2SO_4$ oder kalter Azetonextraktion aus und wandert bei der elektrophoretischen Trennung auf Zelluloseazetat in Barbitalpuffer (pH 8,5) zum positiven Pol. Durch Ultrafiltration wird die Aktivität auf mehrere Fraktionen verteilt, deren Molekulargewichte von mindestens 10 000 bis 100 000 reichen. Vergleiche von Muschel- mit Austernpräparaten zeigen in jedem Fall höhere Aktivitätswerte in Muschelfraktionen als in entsprechenden Austernfraktionen. Die bisher höchsten Werte für die spezifische Aktivität wurden in der hochmolekularen Fraktion von Muschelextrakten gefunden. Die Wirkungsdosis dieses Materials, bei der 50 % der Versuchstiere die „Humping"-Reaktion zeigen, entspricht $0{,}34 \times 10^{-6}$ mg Protein. Neben der „Humping"-Reaktion lösen hochmolekulare Fraktionen von Molluskenextrakten eine Reihe anderer Verhaltensweisen bei Asteroiden aus einschließlich einer Suchreaktion, in deren Verlauf Seesterne sich mehr oder weniger schnell in der Richtung bewegen, in welcher sich die höchste Konzentration des Extrakts befindet. Sowohl im Aquarium wie unter natürlichen Lebensbedingungen im Meer sind auf diese Weise Fortbewegungsgeschwindigkeiten bis zu 8 cm/m beobachtet worden.

## INTRODUCTION

Chemoreception is emerging increasingly as perhaps the single-most important sensory phenomenon governing the lives of invertebrates living in an aqueous environment. That chemoreception is important to (and often dramatically controls) the behavior of insects is well known (Butler 1970) and such knowledge about the chemical control of behavior of various insects, and the specific chemical stimulants involved, has been used successfully to fight and control certain insect species which annually destroy large portions of world food crops. By comparison, little is known about such chemically controlled behavior in marine invertebrates, in spite of the fact that they live in an environment which could be ideally suited for communication by

sensory perception of dissolved substances. In fact, chemoreception in general is probably the least understood of the higher senses (cf. New insight into senses of taste and smell 1972, Carr 1967a, b, Kohn 1961, Nicol 1960, Ramsay 1952, Jahn & Wulff 1950).

A diversity of behavioral characteristics in marine invertebrates is known to be influenced by chemical sensory phenomena (Kohn 1961, Wagner 1905, Copeland 1918, Balke & Steiner 1959, Carr 1967a, b). Some of these behavioral patterns suggesting chemoreceptive mediation are predator and prey recognition (Bullock 1953, Blake 1960), food location by both carnivores and herbivores (Copeland 1918, Frings & Frings 1965), sex differentiation (Coe 1953), and selection of substratum for settling by larva (Birkeland et al. 1971, Scheltema 1961). Many of the overt behavioral sequences displayed in response to stimuli, such as an approaching predator, are well documented (Bullock 1953, Synder & Synder 1971).

The overall picture of chemoreception in marine invertebrates can be divided into three periods. This is possible because of two events which are separated in time by about sixteen years. The first period concerns itself with the investigations carried out prior to 1955. The second period is comprised of the years between 1955 and 1971, and the third period is from 1971 on. The work carried out in the early years of this century, and throughout all of the first period, was mostly observations of behavior patterns induced by the presence of some known stimulant such as food or a predator. This type of work, which is still carried on today (Synder & Synder 1971) is quite valuable and has produced a wealth of observations that invite investigation by more sophisticated chemical methods.

The second period was ushered in with the publication of the results of Loomis' experiments on the chemical control of feeding behavior in the hydra *Hydra littoralis* (Loomis 1955a, b). He demonstrated for the first time that part of the feeding behavior of a marine invertebrate can be controlled by a single identifiable chemical substance, glutathione. The response was reported to be specific for glutathione and could not be elicited by close analogs of that compound (Lenhoff 1968, Lenhoff & Schneiderman 1959). The response to glutathione appeared to be so specific that glutathione was likened to an environmental hormone (Loomis 1955, Lenhoff & Bovaird 1959, 1960). Further, it was suggested that the feeding response of *H. littoralis* be used as a qualitative microbioassay for reduced glutathione (Loomis 1955a, b, Lenhoff & Bovaird 1959).

Because it was now known that a small molecule (glutathione) was the activator for part of the feeding response in *H. littoralis*, many investigators began to search for other small molecules which might induce feeding behavior in marine invertebrates such as other cnidarians (Lenhoff & Schneiderman 1959), gastropods (Carr 1967a, b, Kohn 1961, Brown 1961), and polychaete worms (Mangum & Cox 1966). Some investigators used the now well-documented glutathione-induced feeding response of hydra as a model for examining the mechanisms of chemoreception more closely (Lenhoff 1960, 1968, Lenhoff & Bovaird 1959, 1960, Rushforth & Hofman 1972). During this period, many small molecules were discovered to elicit a feeding response to some extent in a variety of invertebrates, and there is now a voluminous literature associated with this subject. A number of good reviews are available which cover the

activity in this field through 1971 (KOHN 1961, LENHOFF 1968, FORREST 1962, LINDSTEDT 1971). Just a few examples of small molecules which were found to be stimulatory for feeding behavior are betaine and trimethylamine oxide, which are incitants for the spiny lobster *Panulirus* (LAVERACK 1963); leucine, an incitant for the anemone *Haliplanella* (LINDSTEDT 1971); and proline, which incites the hydroid *Cordylophora* (FULTON 1963). In most cases, investigators would randomly select various "small molecule" chemicals which held promise of stimulatory activity because of their known presence in animal tissues, or because of their immediate availability from the stock shelf. Much of the time, this latter method is not a rewarding technique, even if the selected chemical elicits a positive response. For example, trimethylamine oxide (TMO) makes up fully one-third of the excretory products of marine teleosts (BALDWIN 1959), and would, therefore, seem unlikely as a food stimulus for either carnivorous or herbivorous marine invertebrates.

If a chemical stimulant is not naturally available to the respondent, then it is unlikely that it would be of ecological significance. The natural availability of the test substance is, therefore, of primary concern, and when chemical incitants of feeding behavior are to be investigated, the most reasonable method would appear to be to start with a natural food and to isolate and identify a stimulatory chemical from it. This is a technique not often followed, perhaps because of the complexity of the method (CARR 1967a, b).

The third and present phase of the overall picture of chemical control of feeding behavior in marine invertebrates begins with the publication of the results of investigations carried out by MANGUM & COX in April (1971), and by GURIN & CARR, in October (1971). Each of these teams, working independently, and on different species of marine invertebrates, isolated a protein substance which induces part of the feeding behavior in their respective test animals. The polychaete worm, which was the test subject of MANGUM & COX, required relatively high concentrations of the protein, or certain excitatory amino acids, for observable stimulation. This is probably because this animal is a sediment feeder in the wild. GURIN & CARR, on the other hand, isolated and purified, from oyster juice, a single protein which accounts for all of the stimulatory potential that oyster juice possesses for the marine mud snail *Nassarius obsoletus,* an active scavenger. This protein was active at concentrations as low as $2 \times 10^{-10}$ M. For the first time then, a large and complex molecule has been indited as singly responsible for eliciting feeding behavior in an invertebrate. It is important to note here that GURIN & CARR began their work by random selection of various suspected chemicals, in this case proteins, to determine if any of them possessed an innate stimulatory capability. However, after discovering that the human plasma protein albumin, which is certainly not a natural food for *Nassarius obsoletus,* was extremely stimulatory, they then proceeded to extract the active protein from a natural food. In both of the above cases, the protein was found to be glycoprotein, one with a molecular weight of about 20 000 (MANGUM & COX 1971), and the other about 100 000 (GURIN & CARR 1971).

As pointed out by GURIN & CARR (1971), proteins had not been seriously considered as stimulatory substances for several reasons. Probably the most important of these reasons is the high degree of thermal stability exhibited by the mollusk

stimulating factor which they isolated. It is well known that proteins are generally quite susceptible to heat denaturation, and were, therefore, not suspect.

It is obvious then that the present phase of the overall picture of chemoreception in marine invertebrates is concerned with the concept of large complex soluble molecules acting as chemical messengers.

## EXPERIMENTAL

A previous program at this laboratory involving studies of the Pacific starfish *Acanthaster planci* uncovered the interesting fact that part of the feeding behavior of the local starfish *Asterias forbesi* is mediated by chemoreceptive phenomena (BRAUER et al. 1970). Because the active elements appeared to be heat stabile and very water soluble, it was concluded that the stimulatory substances were of low molecular weight.

In light of the recent published findings of GURIN & CARR (1971) that the mollusk stimulating factor was a protein of pronounced heat stability, investigations were initiated to determine if large molecules might also be responsible for initiating that part of the feeding reflex in *A. forbesi* descriptively called the "humping reflex". This is the attack position wherein the animal poses above the shellfish preparative to forcing it open. It is a well known fact that *A. forbesi* is a voracious predator of the American oyster *Crassostrea virginica* and for this reason oyster tissue was selected as the initial source from which to extract possible high molecular weight incitants.

After homogenation and centrifugation of fresh oyster tissue *(C. virginica)* the supernatant solution was subjected to the following procedures to determine if a sequential scheme could be developed for the isolation of active fractions containing large molecules.

### Heat treatment

Homogenized tissue was heated to various temperatures between 40° C and 95° C for periods of 10 to 20 minutes. The mixtures were filtered and tested for activity. It was found that activity was retained after heating to 95° C for 20 minutes.

### Ammonium sulfate extraction

$(NH_4)_2SO_4$ fractionation demonstrated that active constituents could be isolated from a solution that is between 25 % and 50 % saturated with the salt. The recovered fraction was first dialized to remove excess salt before testing on the starfish.

### pH fractionation

Samples were adjusted to either a pH of 4.5, or to a pH of 9.5 using 1N HCl or 1N NaOH. The precipitate was filtered off and the filtrate was adjusted to pH 7.1

using 1N HCl or NaOH. Such treatment resulted in fractions which had no diminished activity.

## Acetone extraction

Samples of the supernatant solution of homogenized tissue were mixed with cold acetone (— 5° C) to various concentrations and centrifuged in the cold. The residue recovered from solutions of between 40 % and 75 % acetone were readily soluble in $H_2O$ and were demonstrably active.

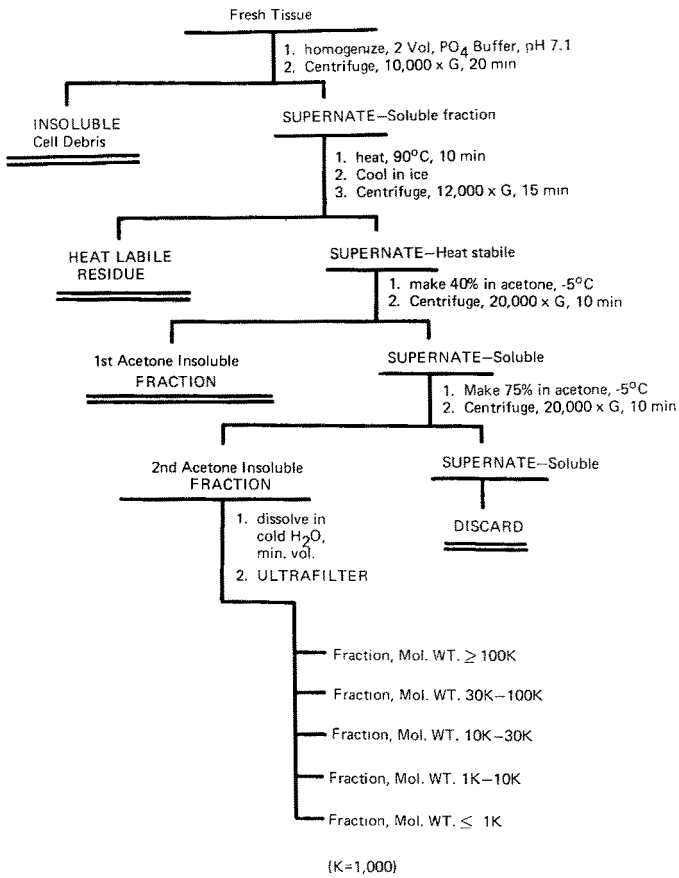


Fig. 1: Fractionation scheme

## Fractionation scheme

The information obtained from the above experiments was utilized to formulate the fractionation scheme shown in Figure 1. After acetone fractionation the experimen-

tal solution was subjected to ultrafiltration through Amicon membranes UM-2, UM-10, PM-30, and XM-100 A. This resulted in fractions with molecular weight ranges of less than or equal to 1000, 1000 to 10 000, 10 000 to 30 000, 30 000 to 100 000 and 100 000 up. This process was also used to fractionate tissue from the clam *Mercenaria mercenaria.*

## Bioassay

A minimum of six animals was used in laboratory assays of each test solution. No one animal was used more frequently than once in six days and no animal was used more than six times for bioassay work. Test animals were starved for three days prior to assays and returned to a laboratory oyster bed until again needed.

Actual testing was performed by placing a starfish in a flat-bottomed dish (20 by 32 cm) in enough fresh seawater to totally submerge him. The animals were then allowed to accommodate to this new environment for fifteen minutes. The test solution was taken up in a 1 ml syringe and gently flowed past the mouth area by use of a 10 cm long, 20 gauge blunt-tipped needle, the last cm of which was bent at a right angle to the shaft. This procedure was performed very carefully so that the needle was never allowed to contact the animal. The flow of solution was controlled to less than 1 ml per minute. A positive response was recorded when a pronounced humping was exhibited in less than 15 seconds. A negative response was recorded if the animal took longer than 60 seconds to respond. Intermediate times, although occurring only infrequently, were thrown out entirely.

In situ bioassays were performed similarly on the ocean floor except that, in addition, attractiveness measurements were also made. In these in situ experiments the test solution was allowed to drift with the current across the animal from about 1 meter upstream. Such tests induced searching activity at speeds of up to 8 cm/min by the starfish.

## RESULTS

Each molecular weight-range fraction was tested at various dilutions with fresh seawater to determine the dilution at which the concentration of protein would be equal to the $ED_{50}$ (Figs 2 and 3). (The $ED_{50}$ is the effective dosage which elicits a positive response in 50 % of the test animals.) Each fraction was then analyzed for protein content and the $ED_{50}$ in mg protein/ml seawater in the test solution was calculated. The protein concentration in mg per ml of extract was obtained from the ratio of absorbance at 280 nm to the absorbance at 260 nm using a Beckman DU spectrophotometer. The results of the bioassays of oyster and clam extracts are plotted as percent response (percentage of animals eliciting a positive response) versus mg protein/ml seawater in the test solution in Figures 4 and 5. Similarly, percent response versus $\mu$l extract/ml seawater in the test solution are the coordinates in Figures 2 and 3 for oyster and clam.

Extract "Activity" is represented as the reciprocal of the $ED_{50}$ and is plotted in a bar graph versus extract fractions for both clam and oyster in Figure 6.

To determine relative heterogeneity, the fractions were subjected to cellulose acetate electrophoresis in barbital buffer at pH 8.6, and ionic strength 0.075. The

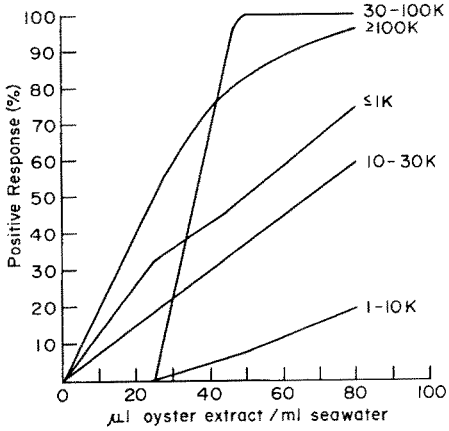


Fig. 2: Responses of starfish to extract volume of oyster tissue

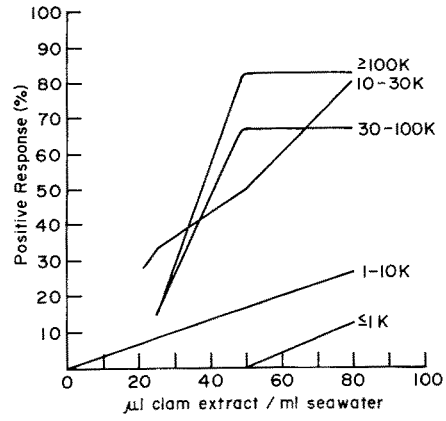


Fig. 3: Responses of starfish to extract volume of clam tissue

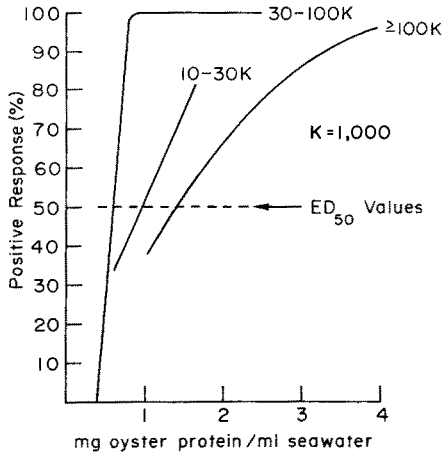


Fig. 4: Responses of starfish to protein content in oyster extracts

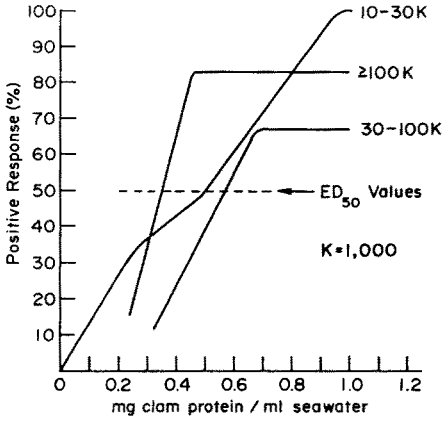


Fig. 5: Responses of starfish to protein content of clam extracts

conditions of electrophoresis were 100 volts and 50 ma for 15 or 20 minutes. The cellulose acetate strips were developed with Ponceau-S dye which reacts with proteins and showed several major bands on the positive side of the strip. Usually these were overlapping or ran together. The highest molecular weight fraction in each case had the best defined band system indicating less heterogeneity than the other fractions.

## DISCUSSION

The starfish *Asterias forbesi* has been shown to be an excellent test subject for studying chemoreception in marine invertebrates. This animal possesses several chemically mediated behavioral responses which are readily observable and rapidly exhibited with a high degree of all-or-none character and are, therefore, readily adaptable to bioassay work in the laboratory. One such response is part of the overall feeding response and is called the humping reflex. It was this chemically induced behavioral response which was the basic bioassay in this investigation.

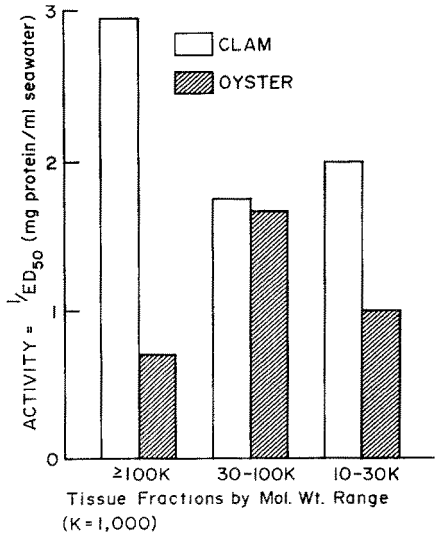


Fig. 6: Excitation activity of tissue fractions by molecular weight range

The primary objective of this work was to determine if large and complex molecules, such as proteins, are involved in the chemoreceptive phenomena of chemically induced feeding behavior in the starfish *A. forbesi*. From the data presented here, it is reasonable to conclude in the affirmative. This is true because: (1) the extraction techniques used in this investigation are standard methods of protein isolation in biochemistry laboratories; (2) electrophoresis demonstrated migration toward the positive electrode in a basic buffer and this is generally characteristic of many proteins; (3) Ponceau-S dye, a dye that is known to stain proteins, was used to develop the bands on the electrophoretic strip; (4) the various fractions were separated by membranes in an ultrafilter cell which retain only large molecules, such as proteins, and (5) the active extracts strongly absorbed light of 280 nm.

From the tissue of two shellfish, the oyster *Crassostrea virginica* and the clam *Mercenaria mercenaria,* protein extracts were prepared and shown to be highly stimulatory. In every case, protein extracts from clam were more active than those from oyster (Fig. 6). In the case of the high molecular weight fraction (100 000 – up), the clam extract was four times as active as the oyster extract.

Assuming a molecular weight of 100 000, and a homogenous protein fraction, the high molecular weight clam extract would be expected to induce a response in 50 % of the test animals at a concentration of $3.4 \times 10^{-6}$ M. This concentration is approximately 136 times greater than the concentration at which GURIN & CARR (1971) reported crystalline human serum albumin stimulated the snail *N. obsoletus* but is approximately the same as those reported by MANGUM & COX (1971) for various incitants of the polychaete worm *D. cuprea*. The protein isolated from oyster extrapallial fluid by GURIN & CARR was 10 000 times more active. This protein was highly purified and homogeneous as demonstrated by its single band upon polacrylamide gel electrophoresis. The protein extracts from clam and oyster tissue prepared in this work very likely were made up of mixtures of many chemicals. Cellulose acetate electrophoresis of the largest molecular weight fraction from both oyster and clam showed the smallest degree of heterogeneity and, therefore, this fraction was the most highly refined.

It is obvious that these fractions must now be purified into component chemicals if any reliable comparisons are to be made with the highly purified protein isolated by GURIN & CARR (1971). Continued intensive investigation of chemoreceptive phenomenon in a wide variety of marine invertebrates is necessary if the knowledge required for eventual identification and classification of the various classes of proteins which are involved in these processes is to be ascertained.

During field and laboratory tests, it was observed that the protein extracts were highly effective in arousing a search reaction in this species of starfish. Typically, the animal would activate the terminal tentacles at the tip of its arm nearest the current-borne extract in an apparent alert response. He would then polarize (i.e., stretch out the nearest arm toward the source of the stimulant), and then proceed to move in that direction. This chemotactic response was clocked at speeds up to 8 cm/min. This is not as fast as the speeds of up to 30 cm/min reported by WHITTLE & BLUMER (1970) in their investigations of chemical attractants of the starfish *Asterias vulgaris*, but this may be a difference between species. It is of interest, however, to note here that a natural substance which can be extracted from natural sources, and does not contaminate or pollute the environment of shellfish, has been shown to act as an artificial attractant to lure starfish toward the source of the substance. Such information may be of importance to the oyster industry where effective pest control is lacking.

## SUMMARY

1. An echinoderm, the starfish *Asterias forbesi*, is described as possessing a chemically mediated behavioral response which is suitable for use as a bioassay in studies on chemoreception.
2. Using *A. forbesi* for bioassays, it was discovered that protein extracts from the clam *Mercenaria mercenaria* and the oyster *Crassostrea virginica* chemically induced the humping reflex in this animal.
3. In every case, the protein extracts from clam were more active (lower $ED_{50}$) than any from oyster. The highest molecular weight-range fraction from clam (100 000

and up) was the most active and had an $ED_{50}$ of $0.34 \times 10^{-6}$ mg protein/ml seawater in the test solution.

4. In laboratory and field tests, the higher molecular weight fractions obtained from ultrafiltration techniques exhibited a pronounced activity as a search-inducing stimulant. The starfish were induced to search at speeds of up to 8 cm/min.

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Author's address: Dr. M. A. HEEB
University of North Carolina at Wilmington
Department of Marine Bio-Medical Research
Wilmington, North Carolina 28401
USA