Chemoreception in Nassarius obsoletus: The Role of Specific Stimulatory Proteins

Abstract. *Proteins in human plasma and oyster fluid induce a strong feeding response in the marine snail* Nassarius obsoletus. *Purified human serum albumin induces a 50 percent positive response at concentrations of 1 to 2 × 10^{-9} molar. Adsorbed fatty acids markedly decrease the effectiveness of albumin. From oyster fluid a major glycoprotein has been isolated which accounts for essentially the entire stimulatory activity of the fluid and is effective at concentrations of approximately 1 to 2 × 10^{-10} molar. These findings provide evidence that specific proteins in extremely low concentrations may play a major role in chemoreception in aquatic animals.*

Chemoreception is known to play an important role in the survival of gastropods and other aquatic invertebrates (*1, 2*). Reports have suggested that chemoreception mediates such diverse phenomena as carrion location by scavengers (*3*), predator avoidance (*4*), prey recognition by predators (*5*), metamorphosis (*6*), plant recognition by herbivores (*7*), and sexual differentiation (*8*). Chemical identification of the stimulatory substances remains to be established in most cases.

The marine mud snail *Nassarius obsoletus* is particularly suitable for studies of chemoreception, as it has been shown to detect substances diffusing from dead animals. Copeland (*3*) observed submerged snails extending their proboscises in response to fish extracts. This proboscis search reaction permitted Carr (*9, 10*) to study stimulatory substances in extracts of shrimp and to demonstrate that solutions of glycine ($10^{-3}M$) and lactate ($5 \times 10^{-4}M$) possessed marked stimulatory activity. Other substances of low molecular weight isolated from shrimp extracts, such as amino acids, betaines, and amines, were relatively inactive. None of the isolated substances singly or in mixtures elicited as strong a response as did the original extracts.

The present work started with the notion that macromolecules such as proteins, lipoproteins, or polysaccharides might play a significant role in chemoreception. Preliminary tests with human plasma revealed that, on a volume basis, it was fully as active as the shrimp extracts reported by Carr in eliciting the proboscis search reaction within 60 seconds (*11*). Upon ultrafiltration through a membrane retaining substances of molecular weight greater than 10,000, it was possible to show that the ultrafiltrate contained less than 10 percent of the stimulatory capacity of either the plasma or the retentate. A second sample of plasma was filtered with a membrane that retained substances of molecular weight 1000 or greater. Again the ultrafiltrate possessed very little stimulatory activity (10 percent) relative to that observed in the retentate or whole plasma (Fig. 1).

Since the major stimulatory activity of plasma resides in the macromolecular fraction, a number of plasma proteins were assayed (*11*) in order to compare the concentrations necessary to induce a 50 percent positive response (ED_{50}). A variety of purified lipoproteins (*12*) ranging from chylomicra to high-density lipoproteins had relatively little stimulatory capacity; ED_{50} values ranged from 6 to 22 μg of protein per milliliter of test solution (*11*). Fibrinogen was also found to be relatively inactive, whereas human serum albumin (Pentex) was highly active (ED_{50} = 2.5 μg of albumin per milliliter of test solution). On the basis of the known concentration of albumin in human plasma (2.8 to 4.5 g/100 ml) it was calculated that albumin alone accounts for some 40 to 70 percent of the total activity of plasma.

Since the albumin preparations were not completely free of fatty acids and other adsorbed substances, a crystalline sample previously treated at *p*H 3.0 with charcoal to remove such adsorbed substances was tested (*13*). Assays of the purified albumin revealed a striking increase in its stimulatory capacity (ED_{50} values of 0.15 to 0.20 μg of albumin per milliliter of test solution). Assuming that plasma contains 2.8 to 4.5 g of albumin per 100 milliliters, it is apparent that this result corresponds to a "recovery" of 5 to 12 times the original activity of whole plasma. A plausible explanation for such an anomalous result involves the reasonable assumption that the albumin in whole plasma contains adsorbed fatty acids as well as traces of ions and other substances which are bound to the albumin. When freed of fatty acids and other adsorbed substances, the concentration of albumin which gives a 50 percent positive response is 1 to 2×10^{-9} molar.

That long-chain fatty acids are strongly adsorbed by albumin has been well established (*14, 15*). Accordingly, a solution of the previously mentioned fatty acid–free albumin was treated with sodium palmitate (2 moles of palmitate per mole of albumin) for 30 minutes at room temperature, diluted appropriately, and tested. As shown in Fig. 2, the stimulatory capacity of the

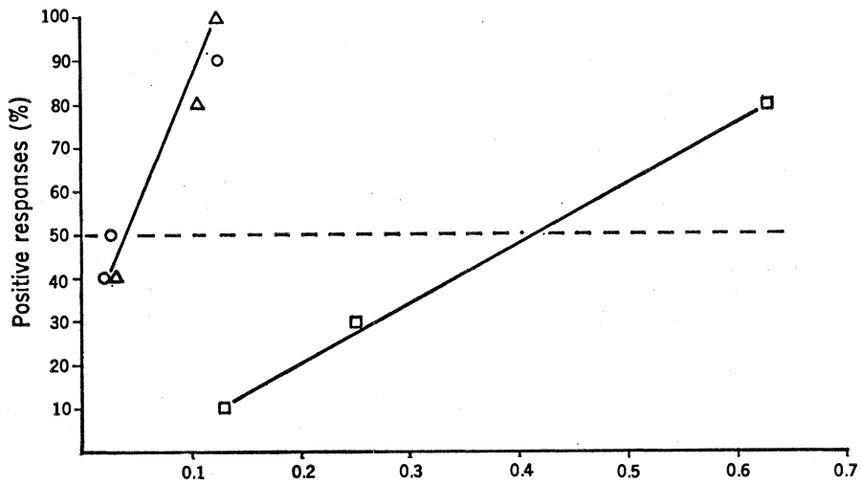

Fig. 1. Response of *N. obsoletus* to human plasma, ultrafiltrate of plasma, and retentate. The abscissa denotes microliters of one of these three substances per milliliter of test solution. All test solutions were prepared from aliquots of the same seawater; ten or more snails were employed at each concentration. The ED_{50} of plasma (○) = 0.03 to 0.04 μl per milliliter of test solution; the ED_{50} of retentate (△) = 0.03 to 0.04 μl per milliliter of test solution; the ED_{50} of ultrafiltrate (□) = 0.4 to 0.5 μl per milliliter of test solution.

albumin was reduced to approximately 5 percent of its original potency by the addition of fatty acid. Since a similar concentration of palmitate, when mixed with another protein (oyster protein, see below), did not diminish its stimulatory potency, it is clear that fatty acid alone, in the concentrations employed, has no apparent inhibitory effect on the chemoreceptors of *N. obsoletus*.

Although the precise sites on the albumin which bind fatty acids have not been identified [at least two strong binding sites are present; see (*15*)] the evidence presented here suggests that such binding sites must be uncovered for maximal stimulatory capacity.

At least 50 percent of the potency is retained when fatty acid–free albumin is heated in a boiling water bath for 1 hour (Fig. 2). This result was surprising. Although no visible flocculation was observed at the concentration employed, it was assumed that the protein had been denatured. In a similar experiment with a very active protein obtained from oyster fluid (see below) little diminution in activity was observed after heating under similar conditions. It is apparent that either the configurational changes produced by denaturation are not sufficient to impair stimulatory capacity, or that heat treatment has produced a modified conformation which is still highly active. In any event these results help to explain why previous investigations have suggested that proteins were not involved in chemoreception, since prolonged heating of crude extracts did not markedly diminish their stimulatory capacity (*9*).

It is of some interest that crystalline bovine serum albumin (free of fatty acids) proved to be very much less active ($ED_{50} = 7.0$ μg per milliliter of test solution) than purified human serum albumin.

A second source of stimulatory proteins was found in oyster fluid obtained by carefully prying apart the valves of fresh oysters (*Crassostrea virginica*). After centrifugation at low temperature, the clear fluid proved to be highly active, and retained its potency after exhaustive dialysis against cold distilled water. Ultrafiltration of the original oyster fluid through a membrane retaining substances of molecular weight 10,000 or greater resulted in no measurable loss of activity in the retentate. Although preliminary electrophoresis on cellulose-acetate strips at *p*H 8.6

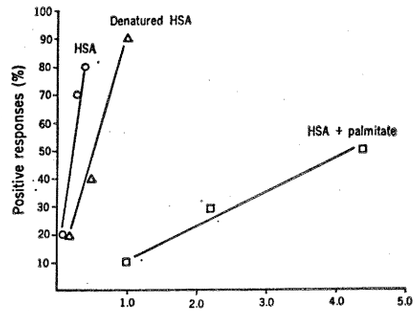

Fig. 2. Response of *N. obsoletus* to human serum albumin (HSA), denatured HSA, and HSA treated with palmitate. The abscissa denotes micrograms of protein per milliliter of test solution. Denatured HSA was prepared by heating 2.175 mg of purified albumin in 3 ml of seawater for 1 hour in a boiling water bath. Palmitate-treated HSA was prepared by adding 0.15 ml of neutralized aqueous solution containing 21.7 μg of palmitate to 3 ml of seawater containing 2.175 mg of purified HSA. The solution was allowed to stand for 30 minutes at room temperature and was then diluted appropriately for bioassay. Denaturation of HSA resulted in 40 to 50 percent diminution of activity, whereas palmitate treatment decreased the response-inducing capacity by approximately 25-fold. In all cases, ten or more snails were employed at each concentration.

indicated the presence of a single major protein fraction, subsequent preparations of oyster fluid were found to contain more than one major protein component when larger quantities were employed, and when electrophoresis was performed with polyacrylamide gel. It is of interest that Kobayashi (*16*) reported the presence of a single protein in oyster extrapallial fluid which had been obtained with minimal trauma. Although considerable care was taken to minimize injury to soft tissues, there was undoubtedly minor contamination of our oyster fluid with other proteins.

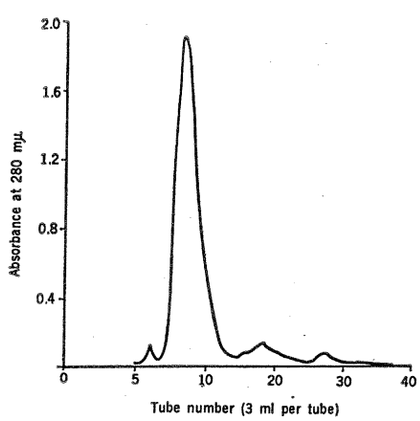

Fig. 3. Chromatographic separation of oyster proteins from fraction No. 4 on G-100 Sephadex. The protein recovered from the major peak proved to be electrophoretically homogeneous, and was found to have an ED_{50} of 0.005 to 0.01 μg/ml.

Two major protein components from oyster fluid were obtained with a diethylaminoethyl (DEAE) column (*17*) after preliminary Millipore filtration, dialysis against cold distilled water, and lyophilization. After removal of several minor fractions by elution of the column with low concentrations of NaCl, the first major protein component (fraction No. 4) was eluted with 0.35*M* NaCl in Veronal buffer. A second major protein (fraction No. 5) was obtained by elution with 0.40*M* NaCl. Fraction No. 4 was then chromatographed on a G-100 Sephadex column from which a single highly purified protein was eluted (Fig. 3) and shown to be homogeneous by vertical polyacrylamide gel electrophoresis (*17*).

The ED_{50} of this purified oyster protein was found to be 0.005 to 0.01 μg per milliliter of seawater. It is a colorless, highly water-soluble glycoprotein containing approximately 6.5 percent carbohydrate (calculated as glucose equivalents by means of the α-naphthol test). Earlier molecular weight determinations of less pure preparations of this glycoprotein suggested a molecular weight of approximately 120,000. Assuming a value of 100,000, the concentration of oyster protein which stimulates a 50 percent positive response in *N. obsoletus* may be calculated to be approximately 1 to 2×10^{-10} molar. Such concentrations correspond to a dilution of 1 : 200,000,000 in seawater.

It is of interest that this particular protein accounts for virtually all of the stimulatory activity (>90 percent) of the original fluid. The other major protein component (No. 5) was considerably less active, and accounted for slightly less than 10 percent of the original activity of the oyster fluid. It was therefore not investigated further. Since the total activity of the freshly obtained oyster fluid may be ascribed to these two proteins, and recalling that no measurable loss of activity was observed after dialysis or ultrafiltration of the original fluid, it is believed that this is the first demonstration of a food-seeking response in a marine animal in which the total stimulatory activity of a biological fluid can be ascribed to isolated and purified chemical entities.

When a solution of the highly active oyster protein was mixed with an excess of palmitate for 30 minutes prior to testing, no diminution of activity was observed. It is presumed that (i) the protein does not bind palmitate and that (ii) free palmitate does not inhibit the feeding response. It should be remembered that palmitate (in similar concentrations) did significantly inhibit the activity of human serum albumin.

As previously mentioned, heating in a boiling water bath (30 to 45 minutes) had very little effect on the stimulatory activity of the oyster protein.

Since *N. obsoletus* is a facultative carrion-feeder, in addition to being a deposit feeder, it is not surprising that there is a nonspecificity for a wide variety of proteins which are stimulatory only at relatively high concentrations. In contrast, fatty acid–free human serum albumin and oyster fluid protein appear to have appropriate configurations and reactive sites which permit them to be remarkably stimulatory. Certainly other very potent proteins will ultimately be found. Hemolymph obtained from the blue crab, *Callinectes sapidus*, is for example, approximately twice as active as human plasma (on a volume basis). If, in addition to *N. obsoletus*, many other marine species respond similarly to highly stimulatory proteins present in blood, exudates of tissues, and so forth, such phenomena can explain why injured species are particularly vulnerable to attack in an aquatic environment.

This highly reactive "binding specificity" of certain proteins for receptor membranes is analogous to the adsorption of protein and peptide hormones by cell surfaces of target organs, and numerous other biological phenomena which suggest that unique proteins, in extremely low concentration, may play an important role as chemical and biological signals. Obviously, further work needs to be done on the chemistry as well as the functional groups and active sites of such proteins. Also, an extensive survey must be conducted to measure the effectiveness of these and other proteins on a variety of marine animals. Such information will be needed in order to appraise the true significance of proteins in chemoreception.

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11. The experimental conditions were identical with those previously described by Carr (*9*). The snails were placed in clean seawater for at least 6 hours prior to testing; the seawater was changed several times. Test solutions were made up with the same sample of seawater. Usually 40 ml of test solution was poured into a petri dish for the assay. Groups of ten snails each were tested at each concentration and the number of positive responses was recorded (four or more extensions of the proboscis within 60 seconds). Positive responses given in control solutions ranged from 0 to 10 percent. Reasonably consistent values for the concentration of proteins (micrograms of protein per milliliter of seawater) necessary to induce a 50 percent positive response (ED_{50}) were obtained. The protein estimations were done by the method of Lowry [see (*18*)].
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17. Chromatographic separation on DEAE column was done with Veronal buffer, *p*H 8.6, ionic strength 0.1, and NaCl solutions referred to in the text. After elution from G-100 Sephadex column, homogeneity of the single protein from fraction No. 4 was established by vertical polyacrylamide gel electrophoresis in tris-EDTA-borate buffer, *p*H 9.2, at 120 ma, 250 volts, for 2.5 hours (EDTA = ethylenediaminetetraacetic acid).
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19. We thank Dr. Paul W. Chun and Miss Beatrice A. Johnston for several highly purified proteins as well as their generous cooperation on the purification and physical properties of oyster fluid proteins. We thank Dr. Waldo R. Fisher for the lipoprotein preparations, and Dr. Michael Roberts for his helpful cooperation.

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Cyclic Adenosine Monophosphate: Function in Photoreceptors

Abstract. *Inactivation of adenylate cyclase in outer segments of retinal photoreceptor cells is proportional to the bleaching of rhodopsin. Membranes of the outer segments also contain a particulate, light-insensitive phosphodiesterase of high specific activity. In electrophysiological experiments, application of cyclic adenosine monophosphate along with a methylxanthine mimics the effects of illumination on the photoreceptor cell of the compound eye of* Limulus.

Adenylate cyclase (cyclase) is an enzyme usually associated with membranes which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic 3′,5′-adenosine monophosphate (cyclic AMP) in response to a variety of peptide hormones or catecholamines (*1*). Cyclic AMP in turn, acting through associated enzyme systems, mediates a number of specialized cellular functions such as melanosome dispersion, release of hormones, and regulation of certain synaptic activities (*2*).

Cyclase is present in retinal photoreceptor structures, where its specific activity is higher than that in any previously described tissue. The cyclase in outer segments of frog rods is inactivated by illumination (*3*). This observation implicates cyclase in photoreceptor function and raises the question of its precise role.

We have investigated this role by studying (i) the correlation between cyclase activity and bleaching of rhodopsin, (ii) phosphodiesterase activity and distribution, and (iii) physiological effects of cyclic AMP in photoreceptors.

Outer segments of bovine retinas adapted to dark were mixed with 45 percent sucrose, and the dense slurry was sonicated to produce a homogeneous suspension of rod disks (*3*). This suspension was pipetted into an ice-cooled Plexiglas chamber (with 1-mm light path) and exposed to tungsten illumination for 1 minute. The intensity of light was varied by adjustment with neutral density filters. After each exposure, 75 μl of the sample was mixed in 400 μl of 1 percent CTAB (cetrimonium bromide) for measurement of optical density at 500 nm, and three 10-μl portions were assayed for cyclase activity by measurement of the amount of labeled cyclic AMP generated from [8-^{14}C]ATP during a 10-

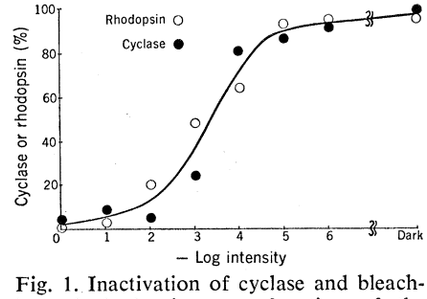

Fig. 1. Inactivation of cyclase and bleaching of rhodopsin as a function of the intensity of illumination. The points shown are the averages for six determinations.