CHEMORECEPTION IN THE MUD SNAIL, NASSARIUS OBSOLETUS. II. IDENTIFICATION OF STIMULATORY SUBSTANCES[1]

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The ecological significance of chemoreception to aquatic gastropods and other aquatic invertebrates is well documented (for reviews, see Kohn, 1961; Hodgson, 1955). However, at the molecular level little is known about the substances which influence the behavior of these animals. Blake (1961) attempted to characterize the oyster metabolites which served as attractants to the predatory oyster drill, *Urosalpinx cinerea.* This work was limited primarily to studies on the physical properties of the attractants and to a series of somewhat inconclusive tests with known compounds. Frings and Frings (1965) studied the physical properties of stimulants which diffused from the food of *Aplysia juliana,* but these studies did not include tests with known compounds. Brown and Noble (1960) and Brown (1961) reported that the gastropod, *Bullia laevissima,* would emerge from its buried position when food was near or when certain quaternary amines were present in sufficient concentrations. No attempt was made to correlate the concentrations of compounds necessary for stimulation with the concentrations available when food was placed in the water. Bailey and Laverack (1963), using electrophysiological techniques, reported that receptors in the osphradium of *Buccinum undatum* were sensitive to *Mytilus* extracts as well as to L-glutamic acid and trimethylamine oxide. This work did not include determinations of the relative concentrations of these compounds in the extracts and no reference was made to any response shown by the intact organism.

Nassarius obsoletus was shown by Carr (1967) to be an extremely suitable animal for studies of chemoreception. This marine gastropod displays a stereotyped response which is convenient for measuring the effectiveness of substances extracted from tissues. Carr showed that the principal response-inducing compounds in shrimp extracts were heat-stable, more soluble in polar than in non-polar solvents, non-volatile, of low molecular weight, stable to oxidation, stable to NH_3, and somewhat acid-labile. Further, it was shown that the principle response-inducers were amphoteric and/or weakly basic compounds which were augmented by some acidic and/or neutral compounds(s).

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The foregoing results have been used as the basis for further experiments on chemoreception in *N. obsoletus*. Ion exchange and paper chromatographic techniques were used to identify and subsequently to quantify compounds in shrimp extracts which possessed the established physical and chromatographic properties. Concurrently, an extensive series of tests was performed to determine the stimulatory capacities of compounds and combinations of compounds which were identified in the extracts. In summary, an attempt has been made to account for the response shown by *N. obsoletus* to shrimp extracts, both in terms of the compounds present and their relative concentrations.

Methods

Maintenance of animals, bioassay procedures, and preparation of shrimp extracts

Specimens of *Nassarius obsoletus* were collected, maintained in the laboratory, and tested as previously described (Carr, 1967).

Chloroform:methanol (2:1) extracts of shrimp (*Penaeus duorarum*) were prepared according to the procedure of Folch, Lees and Sloan Stanley (1957). This procedure yields a two-phase system. In each case the upper aqueous phase was removed, evaporated to dryness in an oven at 50° C., suspended in water (1 ml./1 g. initial shrimp weight), and filtered through a Millipore filter. Upper phase material was used extensively and will be designated shrimp extract.

Elution of stimulatory components from paper chromatograms

Sheets of Whatman No. 1 filter paper were prewashed and shrimp extract was applied as a streak to each sheet. A 12-cm. streak was used for 200 μl. of extract; a 15-cm. streak was used for 300 μl. of extract. After development by the descending method, chromatograms were divided into strips (from defined R_f intervals) at right angles to the direction of solvent flow. Each strip was cut to a point and eluted with glass-distilled water for a 12-hour period. Eluates were evaporated to dryness; the residues were suspended in glass-distilled water and filtered prior to testing.

The following solvent systems were employed: glass-distilled water, butanol: pyridine: water (1:1:1) (Smith, 1960, p. 84), propanol:ammonia:water (6:3:1) (Hanes and Isherwood, 1949), and ethanol: ammonia (95:5) (Smith, 1960, p. 84).

Fractional ion exchange separation

A 0.9 × 145 cm. column of Dowex 50-X2 employed in conjunction with a Beckman Spinco Auto-Analyzer (Model 120) was used for fractionation of a shrimp extract. Six ml. of slightly acidified extract were added to the column and a gradient elution was carried out at a flow rate of 1 ml./minute with pH 3.1 pyridine acetate as the starting buffer and pH 5.0 pyridine acetate as the limit buffer (Nelson *et al.*, 1965). The column jacket temperature was 50° C. Fractions of 6 ml. were collected and 0.5-ml. aliquots were treated with ninhydrin reagent (Moore and Stein, 1954). The optical density at 570 mμ was determined for each aliquot and plotted graphically as a function of the fraction number. Fractions contributing to peaks were pooled, concentrated by rotary evaporation

in vacuo, and each residue was dissolved in 6 ml. of glass-distilled water. Aliquots of each pool were evaporated to dryness in an oven at 50° C. to remove remaining traces of solvent. Residues were redissolved and tested.

Analytical techniques used to identify and quantify compounds in shrimp extracts

Paper chromatography and related techniques. The solvent systems which were used are referred to by the following numbers:

1. Butanol:acetic acid:water (120:30:50) (Smith, 1960, p. 84)
2. Ethanol:ammonia (95:5)
3. Butanol:pyridine:water (1:1:1)
4. Propanol:ammonia:water (6:3:1).

Organic acids were detected with bromcresol green (Smith, 1960, p. 279) or bromphenol blue (Block, Durrum and Zweig, 1958, p. 217). Lactic acid was identified by its R_f values in 4 solvent systems (Nos. 1, 2, 3, and 4).

Betaine and trimethylamine oxide were identified by their reaction with Dragendorff reagent (Bregoff, Roberts and Delwiche, 1953) and by their R_f values in 4 solvent systems (Nos. 1, 2, 3, and 4). Carnitine was tentatively identified by its reaction with Dragendorff reagent and R_f values in two solvent systems (Nos. 1 and 2). Homarine was identified by its UV absorption spectrum, reaction with alkaline alpha-naphthol (Leonard and Macdonald, 1963), and R_f values in two solvent systems (Nos. 1 and 2). Inosine was identified by its UV absorption spectrum and R_f values in 3 solvent systems (Nos. 1, 2, and 3). Urea was identified by its reaction with Ehrlich reagent (Smith, 1960, pp. 193–4) and R_f values in 3 solvent systems (Nos. 2, 3, and 4). Amino acids were identified in the amino acid analyses described below and by two-dimensional chromatography (solvent system No. 1, first dimension: solvent system No. 2, second dimension) using ninhydrin (Smith, 1960, p. 95) as the detection reagent.

Quantitative techniques. Amino acids were analyzed on a Beckman Spinco Auto-Analyzer according to the procedure of Moore, Spackman and Stein (1958) and Spackman, Stein and Moore (1958). Separate analyses were carried out on 100-μl. aliquots of untreated and acid-hydrolyzed (6 N HCl, 20 hours at 110° C.) shrimp extract.

Lactic acid was determined according to the procedure of Barker and Summerson (1941) as modified by Umbreit, Burris and Stauffer (1957, pp. 275–6). A standard curve was prepared using lithium lactate (Amend Drug and Chemical Co.).

Betaine was determined semi-quantitatively by comparing the minimum amount of extracted betaine and betaine-HCl (Calbiochem, A Grade) which could be detected after treatment of chromatograms with Dragendorff reagent. Betaine was also estimated by the visual comparison method of Berry and Cain (1949) as described by Block *et al.* (1958, pp. 86–7).

Preparation of solutions for bioassays of known compounds

For testing a single compound, a concentrated stock solution was prepared in glass-distilled water or in sea water filtered through a Millipore filter; when

necessary the pH was adjusted to 6–8 (determined with pHydrion paper). For testing combinations of compounds, concentrated stock solutions were prepared in sea water filtered through a Millipore filter; and the pH was adjusted as above. A solution of the desired concentration was prepared by pipetting a small volume of stock solution into the appropriate volume of sea water. Stock solutions were prepared immediately prior to testing and kept in ice.

N-Acetylglucosamine (A Grade), L-amino acids (A Grade), betaine-HCl (A Grade), glycogen, and Ca-lactate (B-Grade) were obtained from Calbiochem. Na-ascorbate was obtained from Nutritional Biochemicals. Citric acid (Reagent ACS) was obtained from Matheson Coleman and Bell. Glucose (USP) was obtained from Mallinckrodt. Oxaloacetic acid (99.9% purity) was obtained from Sigma. 2-Aminoethylphosphonic acid was supplied by Dr. L. D. Quin, Department of Chemistry, Duke University.

Statistical treatment of data

Many of the data which follow were analyzed by Potency Probit Analysis employing the computer program of Daum and Givens (1963). This program employs the maximum likelihood procedure of Finney (1962). A brief description of the program was given previously (Carr, 1967). The statistical analyses were carried out in the Duke University Digital Computing Laboratory and in the University of Florida Computing Center. The computer program was provided by Mr. Kenneth Fischler, Biometrician, U. S. Fish and Wildlife Laboratory, Beaufort, N. C.

Results

Elution of stimulatory components from paper chromatograms

Paper chromatography was used as one method for the separation of the components in shrimp extracts. After development of chromatograms in one of a series of four solvent systems, substances from defined sectors were eluted with water and bioassayed. Water was used as the initial solvent system to establish whether or not the stimulatory substances moved near the solvent front. If these substances moved near the front, then water could be assumed to be a satisfactory elutant for subsequent experiments. The other solvent systems (butanol:pyridine:water, propanol:ammonia:water, and ethanol:ammonia) were used to take advantage of the differential mobility shown by many compounds in these solvents.

In each experiment, the response obtained from the combination of all sector eluates (*i.e.*, total extract eluate) was used as a basis for determining the relative effectiveness of eluates from specified sectors of the chromatograms. Figure 1 is a diagrammatic representation of the cumulative results of the elution experiments. The stippled regions on the depicted chromatograms portray the R_f intervals from which substances were eluted which possessed stimulatory capacities approaching those of the total extract eluates. These R_f intervals were as follows: water, R_f 80–100; butanol:pyridine:water, R_f 0–60; propanol:ammonia:water, R_f 40–100; ethanol:ammonia, R_f 0–40.

With the exception of chromatograms developed in water in which the majority of substances closely followed the solvent front, it was necessary to combine sub-

stances eluted from sizable portions of the chromatograms to obtain solutions with stimulatory capacities approaching those of the total extract eluates. Each of these experiments was repeated and similar results were obtained.

Standards of each of the compounds identified in shrimp extracts (see Table II) were chromatographed in butanol:pyridine:water, propanol:ammonia:water, and ethanol:ammonia in order to relate the R_f values of each of these compounds to the R_f intervals from which the most effective eluates were collected during the experiments cited above. The majority of the compounds had R_f values which placed them within the R_f intervals from which the most effective eluates were collected. With butanol:pyridine:water, only isoleucine, leucine, phenylalanine, tryptophan, and tyrosine had R_f values *not included* in the R_f interval 0–60. With

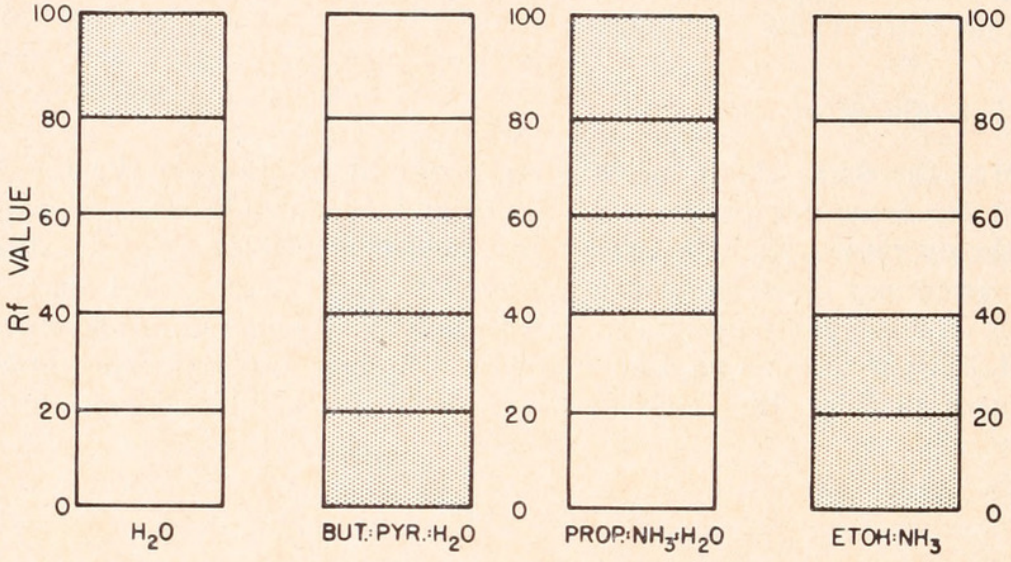

FIGURE 1. Regions of chromatograms from which the principal response-inducing substances in shrimp extracts were eluted. The solvent systems which were employed are given below the diagrammatic paper chromatograms. Stippled regions on the respective chromatograms depict the R_f intervals from which substances were eluted which possessed stimulatory capacities approaching those of the total extract eluates.

ethanol:ammonia, only homarine, isoleucine, leucine, lactic acid, phenlylalanine, trimethylamine oxide, urea, and valine had R_f values not included in the R_f interval 0–40. With propanol:ammonia:water, all of the compounds had R_f values included in the R_f interval 40–100. These findings suggested that the effectiveness of the eluates collected from the R_f intervals cited above was due to the combined effects of a number of compounds.

Fractional separation of shrimp extract on Dowex 50-X2 column

Previous separations of shrimp extracts on ion exchange columns showed that the principal response-inducers were amphoteric and/or weakly basic compounds which were augmented by some acidic and/or neutral compound(s) (Carr, 1967). A more complete fractionation of shrimp extract was achieved by a fractional separation on a column of Dowex 50-X2. An aliquot of each fraction was treated with ninhydrin reagent and the OD_{570} was determined (Fig. 2). The fractions contributing to the OD peaks were pooled and tested (Table I). Fraction 102

(containing fractions 11–18, stippled peak in Fig. 2) was essentially as effective as the total shrimp extract. This conclusion was supported by a Potency Probit Analysis. Three other fractions (101, 104, and 106) were response-inducing at relatively high concentrations. However, the stimulatory capacities of these latter fractions were very low when compared with the capacity of fraction 102.

Table II summarizes the distribution of the extract components identified in fraction 102 and in other preparations obtained by ion exchange separations which were reported previously (for details of separations of shrimp extracts on columns of Rexyn 102 and Dowex 50W-X8, see Carr, 1967). The water effluent from a

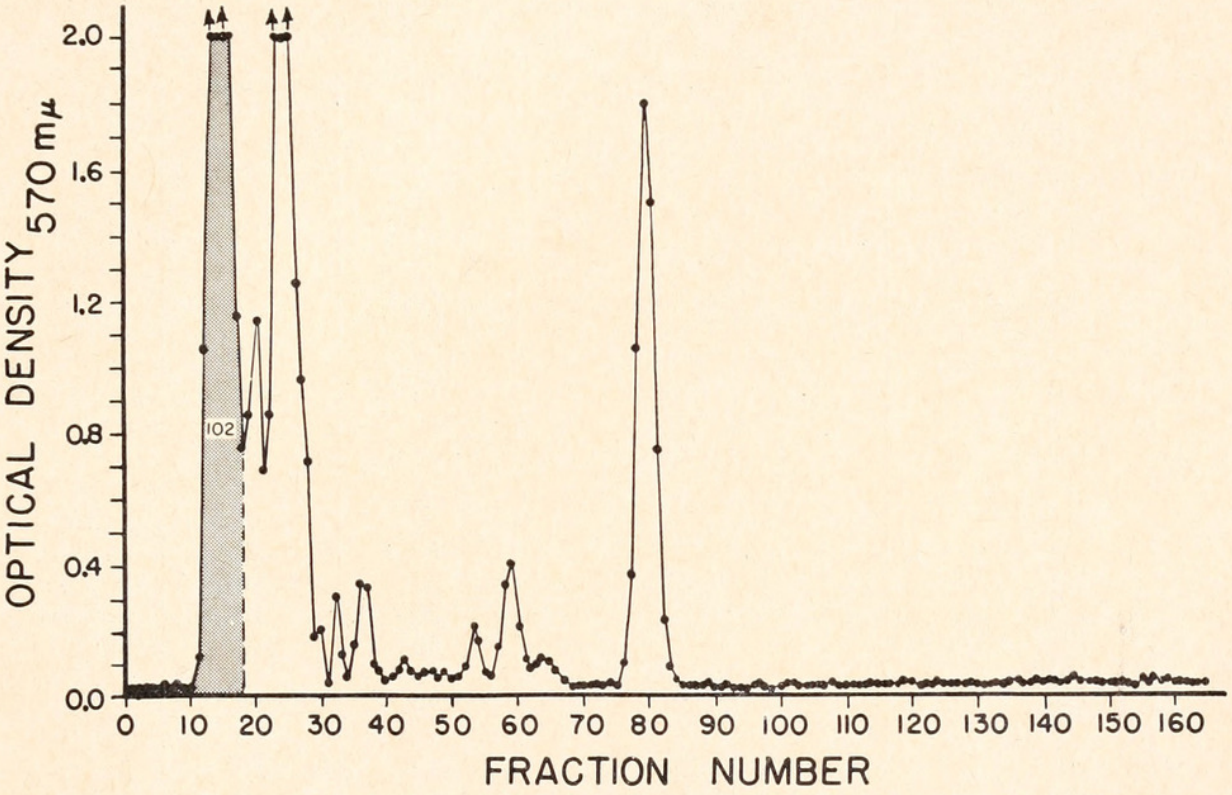

FIGURE 2. Fractionation of a shrimp extract on a column of Dowex 50-X2. Fractions of 6 ml. were collected and aliquots of each were treated with ninhydrin reagent. The optical density at 570 mμ was determined for each aliquot and is plotted as a function of the fraction number. Fractions contributing to peaks were pooled and tested (see Table I).

column of Rexyn 102 contained all of the compounds identified in shrimp extracts except "carnitine," trimethylamine oxide (TMO), and lysine. Bioassays had revealed that this effluent possessed a stimulatory capacity comparable to that of the total extract. These results implied that carnitine, TMO, lysine, or other such basic compounds, which might be present in the extract, did not contribute appreciably to the stimulatory capacity. The 2 N-NH_3 eluate from a column of Dowex 50W-X8 contained all of the compounds identified in the extracts except lactic acid, taurine, and possibly lysine. Bioassays had revealed that this eluate was somewhat less effective than the total extract. The only compounds identified in the water effluent from the column of Dowex 50W-X8 were lactic acid and taurine. Bioassays had revealed that this effluent was only effective at high concentrations when tested alone, but that a combination of the water effluent and the

2 N-NH_3 eluate was as effective as the total extract. These results implied that, although lactate, taurine, and/or some other compound(s) in the water effluent were not the principal source of the extract's stimulatory capacity, they were nevertheless contributors to this capacity. Finally, fraction 102 lacked the following components of the extract: "carnitine," histidine, isoleucine, leucine, lysine, methionine, phenylalanine, TMO, tryptophan, tyrosine, and valine. Bioassays had revealed that this Fraction alone was essentially as effective as the total extract

TABLE I

Responses of N. obsoletus to total shrimp extract and to extract constituents present in fractions collected from column of Dowex 50-X2

Material tested	Column fractions pooled*	Concentration tested (μl./40 ml.)	No. snails tested	% Response
Total shrimp extract		0	20	10
		1	20	15
		2	10	70
		5	20	85
		10	20	70
		20	10	100
Fraction 101	1–10	20	10	30
		60	10	50
102	11–18	2	10	60
		5	30	73
		10	10	60
		20	10	90
103	19–21	60	10	0
104	22–29	20	10	30
		60	10	60
105	30–34	60	10	10
106	35–40	20	10	20
		60	10	40
107	41–50	60	10	20
108	51–56	60	10	0
110	63–75	60	10	10
111	76–85	60	10	10
112	86–115	60	10	10
113	116–165	60	10	20

* The fractions from the Dowex 50-X2 column which contributed to OD_{570} peaks (see Fig. 2) were pooled together. These pools of fractions are referred to as "Fractions" in left hand column of Table.

(see Table I). This suggested that the extract's stimulatory capacity was attributable to a "nucleus" of compounds (seemingly represented in Fraction 102) which did not include the members of the list of compounds given immediately above. These results and their implications were investigated as a major portion of the final phase of the problem which is reported later in the Results.

Quantitative analyses of compounds present in shrimp extracts

Analyses were made to determine the concentrations of the individual amino acids, lactate, and betaine in shrimp extracts. The analyses permitted the testing

of artificial solutions which contained various (or all) of these compounds in the same relative concentrations as they occurred in the extracts.

Amino acid analyses. The results of analyses of untreated and acid-hydrolyzed extract are given in Table III. Nineteen amino acids were identified in the untreated extract and 18 were measured quantitatively. Of the latter amino acids, glycine alone accounted for approximately 55% of the total μmoles. Together,

TABLE II

Distribution of identified shrimp extract components in stimulatory ion exchange preparations

Compounds identified in shrimp extracts	H_2O effluent from Rexyn 102 column	H_2O effluent from Dowex 50 column	2 N-NH_3 eluate from Dowex 50 column	Fraction 102 from Dowex 50 column
Alanine	+		+	+
Asparagine	+		+	+
Aspartic acid	+		+	+
Betaine	+		+	+
"Carnitine"*			+	
Glutamic acid	+		+	+
Glutamine	+		+	+
Glycine	+		+	+
Histidine	+		+	
Homarine	+		+	+
Inosine	+		+	+
Isoleucine	+		+	
Leucine	+		+	
Lactic acid	+	+		+
Lysine			?	
Methionine	+		+	
Phenylalanine	+		+	
Proline	+		+	+
Serine	+		+	+
Taurine	+	+		+
Threonine	+		+	+
Trimethylamine oxide			+	
Tryptophan	+		+	
Tyrosine	+		+	
Urea	+		+	+
Valine	+		+	
UV-A**	+		+	+

* This compound was tentatively identified as carnitine.
** Unidentified UV-absorbing compound.

glycine, proline (18%), taurine (11%), and alanine (10%) accounted for approximately 94% of the total amino acids in the untreated extract.

Lactic acid analyses. Lactic acid was the only organic acid identified in shrimp extracts. The results of duplicate analyses of lactic acid in two shrimp extracts were as follows:

	μg. lactate/μl. extract
Extract 1	3.95
Extract 2	3.10

The average of these two values (*i.e.* 3.53 μg./μl.) was used in the preparation of stock solutions referred to later in the Results.

Betaine analyses. Semi-quantitative determinations of betaine in shrimp extracts revealed that there were approximately 10 μg. (expressed as betaine-HCl) per μl. of the extracts. This value of 10 μg./μl. was used in the preparation of stock solutions referred to later in the Results.

TABLE III

Amino acids present in shrimp extract

Amino acid	Untreated shrimp extract (μmoles/100 μl.)	Acid-hydrolyzed shrimp extract (μmoles/100 μl.)
Alanine	0.866	0.915
Arginine	—	0.667
Asparagine	0.058*	—
Aspartic acid	0.014	0.072
Glutamic acid	0.049	0.454
Glutamine	0.405*	—
Glycine	4.72	5.34
Histidine	0.064	0.118
Isoleucine	0.064	0.087
Leucine	0.084	0.112
Lysine	0.070	0.033
Methionine	0.083	0.094
Phenylalanine	0.068	0.073
Proline	1.58	1.89
Serine	0.111**	0.111
Taurine	0.938	1.04
Threonine	0.113**	0.113
Tryptophan	Trace	—
Tyrosine	0.052	?
Valine	0.110	0.182

* Calculated from difference between pre- and posthydrolysis values of glutamic acid or aspartic acid.

** Post-hydrolysis value. In analysis of untreated extract, serine, threonine, asparagine, and glutamine came off the column almost simultaneously and their individual concentrations could not be calculated. After hydrolysis, serine and threonine came off as separately definable peaks. Assignment of post-hydrolysis values to amino acids in the untreated extract is subject to a small error but nevertheless provided "approximately correct" values which were used to prepare solutions described later in the Results.

Bioassays of individual compounds

Compounds were selected for testing on the basis of their inclusion in one or more of the following categories: (1) Positive or tentative identification in shrimp extracts. (2) Ubiquitous occurrence at relatively high concentrations in marine animals. (3) Reported to induce feeding responses from other animals. (4) Structural relationship to compound(s) observed to induce responses in *N. obsoletus*.

The most obvious group of compounds identified in shrimp extracts were the amino acids. These identified amino acids were tested individually at concentra-

tions of 10^{-3}, 10^{-4}, and 10^{-5} *M* (unless otherwise specified). Only glycine induced the PSR. The results of tests with glycine are given in Table IV. The concentrations of glycine shown in the Table are all more than 100 times the glycine concentration present in dilutions of shrimp extracts which were stimulatory. Glycine alone was ineffective at the concentrations present in solutions of diluted extract which were capable of marked stimulation.

TABLE IV

Responses of N. obsoletus to known compounds

Compounds identified in shrimp extracts				Compounds not identified in shrimp extracts			
Compound tested	Concentration tested (*M*)	No. snails tested	% response	Compound tested	Concentration tested (*M*)	No. snails tested	% response
Glycine	5.9×10^{-4}	20	25	N-Acetyl-D-glucosamine	1×10^{-4}	10	20
	1.2×10^{-3}	20	45		5×10^{-4}	20	55
	2.4×10^{-3}	20	60		1×10^{-3}	10	50
Lactate	1.0×10^{-4}	20	15	2-Aminoethylphosphonic acid	5×10^{-5}	10	0
	2.5×10^{-4}	20	45		1×10^{-4}	10	40
	5.0×10^{-4}	20	65		5×10^{-4}	10	50
Betaine	1.6×10^{-4}	10	10	Pyruvate	2.5×10^{-4}	20	35
	8.0×10^{-4}	20	25		5.0×10^{-4}	20	50
	1.6×10^{-3}	20	25		1.0×10^{-3}	20	65
	3.2×10^{-3}	10	10				
				Glycogen	0.03 mg./ml.	20	15
					0.06 mg./ml.	20	55
					0.24 mg./ml.	10	80
				D-Glucose	1×10^{-4}	10	0
					1×10^{-3}	10	0
					5×10^{-3}	20	30
					1×10^{-2}	20	30
				Ascorbate (Na)	1×10^{-5}	10	10
					1×10^{-4}	20	20
					1×10^{-3}	10	30
				Citric acid	1×10^{-5}	10	0
					1×10^{-4}	20	30
					5×10^{-4}	20	20
				Oxaloacetic acid	1×10^{-5}	10	0
					1×10^{-4}	20	25
					1×10^{-3}	20	30

Lactate [L (+) Ca-lactate] stimulated the PSR (Table IV) but only at high concentrations relative to extract. The concentrations of lactate shown in the Table are all more than 20 times the lactate concentration present in stimulatory dilutions of shrimp extracts. As with glycine, lactate was ineffective at the concentrations present in solutions of diluted extract which were capable of marked stimulation. Preliminary tests with D(−) lactate revealed that it also was

response-inducing but no extensive tests were carried out to compare the stimulatory capacities of the two optical isomers.

Pyruvate (Na) also induced the PSR (see Table IV); it was not identified in shrimp extracts but is mentioned here because of its structural relationship to lactate. A comparison of the results with pyruvate and lactate reveals that the two compounds possessed quite similar stimulatory capacities.

TABLE V

Compounds which were non-stimulatory to N. obsoletus

Amino acids, amines, and related compounds	Range of concentrations tested (M)	Organic acids	Range of concentrations tested (M)
Acetyl choline-Cl	5×10^{-3}–5×10^{-6}	Acetate-Na	5×10^{-4}–10^{-4}
L-Alanine*	10^{-3}–10^{-5}	Fumaric acid	10^{-3}–10^{-5}
γ-Aminobutyric acid	10^{-3}–10^{-5}	DL-beta-hydroxy-butyrate-Na	10^{-3}–10^{-5}
Ammonium-Cl	10^{-3}–10^{-5}	Malic acid	10^{-3}–10^{-5}
L-Arginine-HCl	10^{-3}–10^{-5}	Malonic acid	10^{-3}–10^{-5}
L-Asparagine*	10^{-3}–10^{-5}	Succinic acid	10^{-3}–10^{-5}
L-Aspartic acid*	10^{-3}–10^{-5}		
DL-Carnitine-HCl*	10^{-3}–10^{-5}		
Choline-Cl	10^{-3}–10^{-5}	*Carbohydrates*	
L-Citrulline	10^{-3}–10^{-5}		
Deoxycarnitine-HCl	10^{-3}–10^{-5}	D-Glucosamine[00]	5×10^{-4}–10^{-4}
L-Glutamic acid*	10^{-3}–10^{-5}	D-Mannose	10^{-2}–10^{-4}
L-Glutamine*	10^{-3}–10^{-4}	Trehalose	10^{-2}–10^{-5}
Glutathione**	10^{-3}–10^{-5}		
Glycylglycine	10^{-3}–10^{-5}	*Miscellaneous*	
L-Histidine-HCl*	10^{-3}–10^{-5}		
Homarine-SO_4*	10^{-4}–10^{-6}	Adenosine-5-mono-phosphate[000]	10^{-4}–10^{-6}
L-Isoleucine*	10^{-3}–10^{-5}	Adenosine-5-tri-phosphate[000]	5×10^{-4}–5×10^{-5}
L-Leucine*	10^{-3}–10^{-5}	Inosine*	10^{-4}–10^{-6}
L-Lysine-HCl*	10^{-3}–10^{-5}	Inosine-5-mono-phosphate	10^{-4}–10^{-6}
L-Methionine*	10^{-3}–10^{-5}	Riboflavin[00]	10^{-4}–10^{-7}
Nicotinic acid[00]	10^{-3}–10^{-5}	Uridine-5-mono-phosphate	10^{-4}–10^{-6}
L-Phenylalanine*	10^{-3}–10^{-5}		
O-Phosphoethanolamine	10^{-3}–10^{-5}		
L-Proline*[0]	10^{-3}–10^{-5}		
Sarcosine-HCl	10^{-3}–10^{-5}		
L-Serine*	10^{-3}–10^{-5}		
Taurine*	10^{-3}–10^{-5}		
L-Threonine*	10^{-3}–10^{-5}		
Trimethylamine-HCl	10^{-3}–10^{-5}		
Trimethylamine oxide*	10^{-3}–10^{-5}		
L-Tryptophan*	10^{-3}–10^{-5}		
Urea*[00]	10^{-3}–10^{-5}		
L-Valine*	10^{-3}–10^{-5}		

* Compounds identified, or tentatively identified, in shrimp extracts.

** Glutathione (10^{-3}–10^{-6} M) induces a feeding response in *Hydra littoralis* (Loomis, 1955) and *Physalia physalis* (Lenhoff and Schneiderman, 1959).

[0] Proline (10^{-3}–10^{-6} M) induces a feeding reaction in *Cordylophora lacustris* (Fulton, 1963).

[00] Glucosamine (10^{-1}–10^{-5} M), nicotinic acid (10^{-2}–10^{-11} M), riboflavin (10^{-5}–10^{-7} M), and urea (10^{-1}–10^{-3} M) induce ingestion of agar cubes in *Hydra pseudoligactis* (Forrest, 1962).

[000] Adenosine monophosphate (10^{-2}–10^{-5} M) and adenosine triphosphate (10^{-4}–10^{-5} M) induce gorging in the mosquito, *Culex pipiens* (Hosoi, 1959).

Betaine (HCl) was mildly stimulatory but responses of only 25% were obtained with concentrations greater than 100 times the betaine concentration in dilutions of shrimp extracts which were stimulatory. No correlation was apparent between the concentration of betaine tested and the percentage of snails responding.

The relationships between the effective concentrations of shrimp extract and the effective concentrations of glycine, lactate, and betaine are presented graphically at the end of the Results (see Fig. 4).

None of the other compounds identified in shrimp extracts was effective at the concentrations tested. These compounds, and others which were ineffective, are given in Table V.

2-Aminoethylphosphonic acid, N-acetyl-D-glucosamine, and glycogen were not identified in shrimp extracts but were found to possess marked stimulatory capacities (Table IV). D-Glucose, ascorbate (Na), citric acid, and oxaloacetic acid were also not identified in shrimp extracts but were found to be mildly stimulatory (Table IV).

Bioassays of combinations of compounds

Glycine, betaine and lactate were the only compounds identified in shrimp extracts which were stimulatory when tested individually. However, the concentrations of glycine, betaine and lactate in the extracts were much too low to account for the stimulatory capacities of the extracts themselves. The cumulative results of the elutions from paper chromatograms, the separations on ion exchange columns, and the assays of individual compounds revealed that stimulation by shrimp extracts was not due to a single compound and yet not dependent on the presence of every compound in the extracts. Furthermore, stimulation was attributable to amphoteric and/or weakly basic compounds which were augmented by some acidic and/or neutral compound(s).

The constituents of Fraction 102, obtained from the fractional separation of shrimp extract on a column of Dowex 50-X2, possessed a stimulatory capacity comparable to that of the total extract (see Table I) and included a group of identified compounds satisfying the criteria cited above (see Table II). Mixtures of compounds were tested in an attempt to account for the effectiveness of the extracts. Decisions concerning the choice of pertinent compounds to test stemmed from the cumulative results of the many prior tests and yet had as their central focus the results of the fractional separation cited above.

Table VI gives the composition of stock solutions which were used for a number of the bioassays of combinations of compounds. The concentrations given in the Table are the same as the concentrations determined in shrimp extracts (*i.e.*, one μl. of stock solution contains an amount of each component which is comparable to the amount in one μl. of extract).

The amino acids in Solution A, and the betaine (Table VI), were (1) all present in fraction 102, (2) all present in the water effluent from a column of Rexyn 102, (3) all, except taurine, present in the 2 N-NH_3 eluate from a column of Dowex 50W-X8 (taurine was present in the water effluent), and (4) all present in the R_f intervals, 0–40 (solvent, ethanol:NH_3), 40–100 (solvent, propanol:NH_3:water), and 0–60 (solvent, butanol:pyridine:water). Lactate was (1) present in fraction 102, (2) present in the water effluent from a column of Rexyn 102, (3) present in

the water effluent from a column of Dowex 50W-X8, and (4) present in the aforementioned R_f intervals in the solvents propanol:NH_3:water and butanol: pyridine:water, but had an R_f of 52 in ethanol:NH_3.

The following studies were carried out over a 3-month period during the summer (1965) because of indications of a seasonal variation in responsiveness (unpublished personal observations). It was apparent that snails collected during the warm months were somewhat more responsive than snails collected during the cold months.

Tests of Solution A, betaine, and lactate, and glycine, betaine, and lactate. Figure 3 contains the results of tests of Solution A (glycine + 9 other amino acids), betaine, and lactate, and of glycine, betaine, and lactate. The combination of Solution A, betaine, and lactate was very effective and possessed a stimulatory capacity considerably greater than was attributable to the concentrations of glycine,

TABLE VI

Composition of stock solutions used for bioassays of combinations of compounds

Stock solution	Compounds included	Concentration (mg./ml.)
Solution A (amino acids)	L-Alanine	0.77
	L-Asparagine	0.09
	L-Aspartic acid	0.02
	L-Glutamic acid	0.07
	L-Glutamine	0.59
	Glycine	3.54
	L-Proline	1.82
	L-Serine	0.12
	L-Taurine	1.18
	L-Threonine	0.14
Betaine solution	Betaine-HCl	10.0
Lactate solution	L(+) Ca-Lactate	5.92

betaine, and lactate which were present in it. With the exception of glycine, the amino acids in Solution A were *not* effective when tested individually even at high concentrations (10^{-3} *M*). However, when combined with glycine, betaine, and lactate, these amino acids made a marked contribution to the stimulatory capacity. The Solution A-betaine-lactate combination was approximately 10–13 times as effective as either lactate or glycine-betaine-lactate and approximately 37 times as effective as glycine. The glycine-betaine-lactate combination was no more effective than lactate alone. Lactate and glycine-betaine-lactate were both significantly more effective than glycine.

Tests of other combinations of Solution A, betaine, and lactate. In an attempt to gain insight into the relative importance of betaine and lactate in the Solution A-betaine-lactate combination, solutions were tested which lacked betaine and/or lactate. The results are given in Table VII. Solution A was markedly less effective than combinations of Solution A with betaine or lactate. At concentrations of 250, 500, and 1000 μl./40 ml., Solution A induced responses of 40%, 50%,

and 40%, respectively, whereas at concentrations of only 25, 50, and 100 μl./40 ml., Solution A-lactate induced responses of 42%, 60%, and 55%, respectively, and Solution A-betaine induced responses of 20%, 43%, and 47%, respectively. The Solution A-lactate combination was seemingly more effective than the Solution A-betaine combination; however, a Potency Probit Analysis showed that neither of the latter combinations was significantly less effective than the Solution A-

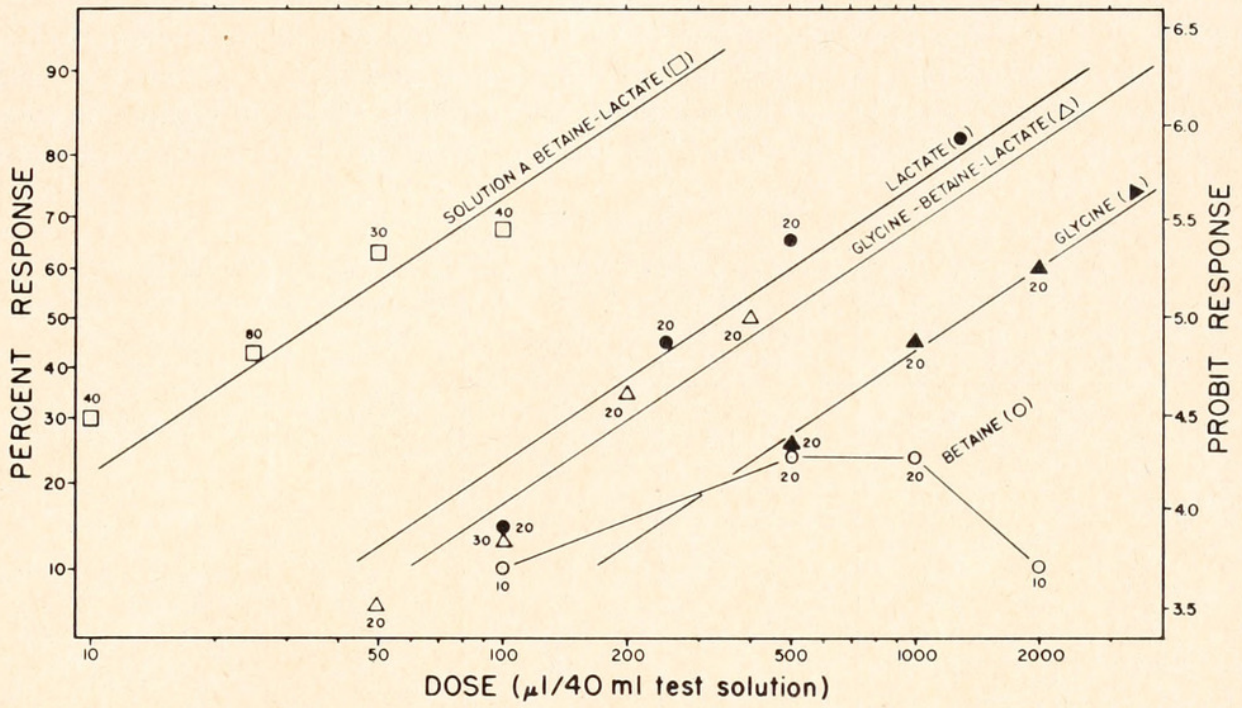

FIGURE 3. Results of bioassays of certain compounds identified in shrimp extracts. Per cent response (left ordinate) and probit response (right ordinate) are plotted as a function of dose (abscissa-$\log_{10}$ scale). The experimental dosage-response values are indicated by symbols; numbers next to symbols indicate numbers of snails tested. Regression lines were drawn from computed effective dose values obtained by Potency Probit Analysis. No regression line was computed for results obtained with betaine. The potencies (and 95% confid. lims.) given below were computed with respect to the potency of Solution A-betaine-lactate:

Glycine-betaine-lactate	Upper lim.	0.141
	Potency	0.079
	Lower lim.	0.036
Lactate	Upper lim.	0.206
	Potency	0.109
	Lower lim.	0.056
Glycine	Upper lim.	0.052
	Potency	0.028
	Lower lim.	0.014
Betaine	Not computed	

betaine-lactate combination. Nevertheless, the difference in the calculated potencies of Solution A-betaine-lactate and Solution A-betaine (no lactate) was nearly significant; the upper and lower 95% confidence limits were 1.19 and 0.22, respectively (see Table VII). During the bioassays this difference seemed very real and it was felt that the combination which *excluded* lactate was *less* effective than either Solution A-betaine-lactate or Solution A-lactate. The role of betaine was

uncertain; Solution A-betaine was considerably more effective than Solution A alone but Solution A-lactate (no betaine) was essentially as effective as Solution A-betaine-lactate.

Tests of Solution A, betaine, and lactate plus additional compounds identified in shrimp extracts. Histidine, isoleucine, leucine, lysine, methionine, phenylalanine, trimethylamine oxide, tryptophan, tyrosine, urea, and valine were other compounds identified in shrimp extracts. In order to determine whether these compounds contributed to the response-inducing capacity of the extracts, these compounds were incorporated into a single stock solution and tested in combination with Solution A-betaine-lactate. The relative concentration of each compound was equal to the concentration determined in shrimp extracts. Incorporation of these

TABLE VII

Responses of N. obsoletus to Solution A, Solution A-betaine, and Solution A-lactate

Solution A* (μl./40 ml.)	Betaine* (μl./40 ml.)	Lactate* (μl./40 ml.)	No. snails tested	Per cent response	Potency** (95% confid. lims.)
125	—	—	10	20	Not computed
250	—	—	20	40	
500	—	—	20	50	
1000	—	—	10	40	
25	25	—	10	20	Up. lim. 1.198
50	50	—	30	43	Potency 0.489
100	100	—	30	47	Low. lim. 0.219
200	200	—	30	73	
10	—	10	40	35	Up. lim. 2.124
25	—	25	60	42	Potency 1.030
50	—	50	40	60	Low. lim. 0.509
100	—	100	40	55	

* Stock solutions prepared as given in Table VI.

** Potencies (and confid. lims.) computed with respect to the potency of Solution A-betaine-lactate. Data from tests of the latter combination are given in Figure 3.

additional compounds yielded a solution which was no more effective than a solution containing only Solution A, betaine, and lactate. This conclusion was supported by a Potency Probit Analysis. The implication that histidine, isoleucine, leucine, lysine, methionine, phenylalanine, trimethylamine oxide, tryptophan, tyrosine, and valine were not among the principal contributors to the stimulatory capacity of the extracts was suggested initially by the fact that these compounds were not identified in fraction 102 (see Table II).

Summary—Relative effectiveness of shrimp extract and certain components in shrimp extract. Figure 4 permits a more adequate visualization of the approach made to the response-inducing capacity of shrimp extract as a result of the systematic incorporation of extract components. The complete response-inducing capacity of the extract was not attained with the combinations of compounds tested; the extract was approximately 6 times as effective as the Solution A-betaine-lactate combination. Nevertheless, the results very strongly implied that the extract's

stimulatory capacity stemmed from a group of compounds. Solutions containing only Solution A (10 amino acids), glycine, betaine, *or* lactate (or glycine-betaine-lactate) were considerably less effective than Solution A-betaine-lactate. The effectiveness of the Solution A-betaine-lactate combination was *not* simply a function of the presence of more organic molecules; actually there were considerably fewer molecules present in the stimulatory solutions produced from combining Solution A, betaine, and lactate than in the less effective solutions containing only certain of these constituents.

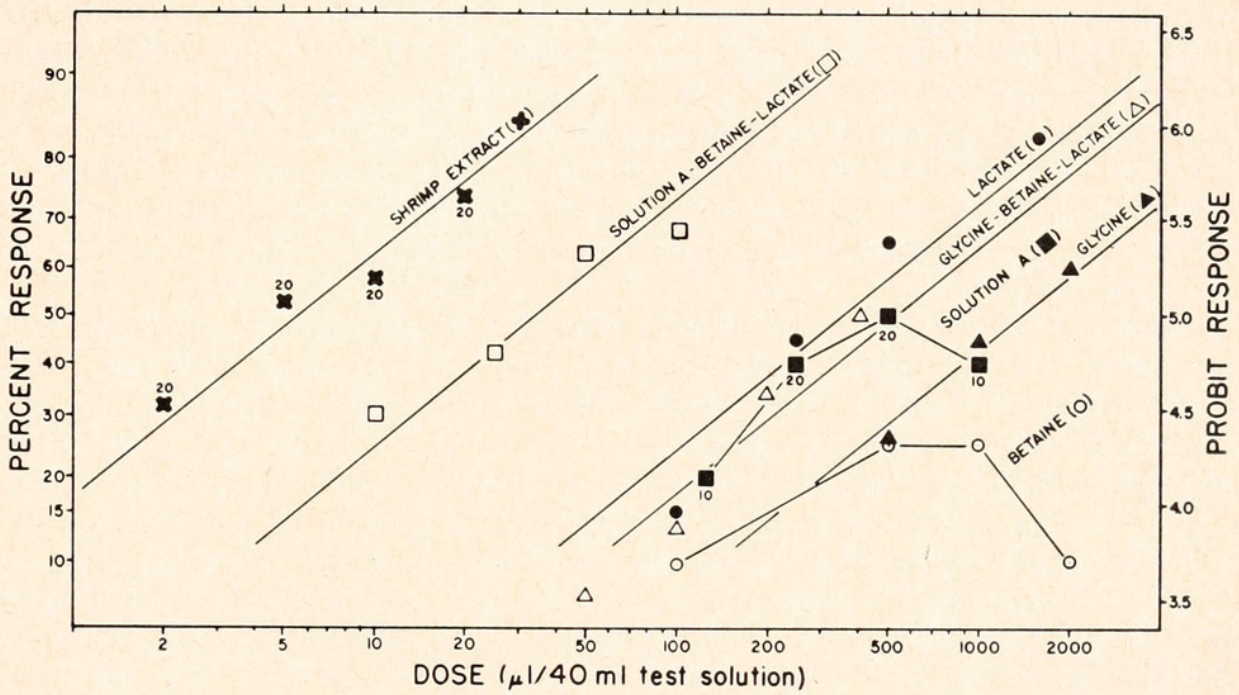

FIGURE 4. Responses given by *N. obsoletus* to a shrimp extract and to certain compounds identified in shrimp extracts. Per cent response (left ordinate) and probit response (right ordinate) are plotted as a function of dose (abscissa-$\log_{10}$ scale). The experimental dosage-response values are indicated by symbols. Numbers next to symbols for tests of shrimp extract and Solution A indicate numbers of snails tested; numbers of snails tested with other preparations were given in Figure 3. Regression lines were drawn from computed effective dose values obtained by Potency Probit Analysis. No regression lines were computed for results obtained with Solution A or betaine. The potencies (and 95% confid. lims.) given below were computed with respect to the potency of shrimp extract:

Soln. A-betaine-lactate	Upper lim.	0.313
	Potency	0.166
	Lower lim.	0.084
Lactate	Upper lim.	0.035
	Potency	0.016
	Lower lim.	0.006
Glycine-betaine-lactate	Upper lim.	0.023
	Potency	0.011
	Lower lim.	0.004
Glycine	Upper lim.	0.009
	Potency	0.004
	Lower lim.	0.002
Soln. A	Not computed	
Betaine	Not computed	

Discussion

By using the proboscis search reaction of *Nassarius obsoletus* as the criterion of response, it has been possible to describe three aspects of stimulation by tissue extracts: (1) individual stimulatory compounds were identified; (2) the effectiveness of these compounds was measured as a function of their concentrations in the extracts; and (3) mixtures of compounds were shown to be more effective than the individual compounds in the mixtures.

The approach taken by previous workers on related problems with aquatic invertebrates was confined primarily to the identification of specific stimulatory compounds (*cf.* Loomis (1955) with *Hydra*; Lenhoff and Schneiderman (1959) with *Physalia*; Brown (1961) with *Bullia*; and Fulton (1963) with *Cordylophora*). No attempts were made to show that the concentration of the identified response-inducer(s) in tissue preparations was sufficient to account for the responses observed to the preparations themselves. Moreover, in the present study a chemically mediated response has been shown to involve a group of compounds whose members complement one another in a manner unsuspected on the basis of tests of these individual members.

All of the compounds included in the studies of stimulation by combinations of compounds were identified constituents of shrimp extracts and are representative of the compounds found in the tissues of other marine animals. Free amino acids and betaine occur in high concentrations in marine invertebrates (Awapara, 1962; Laverack, 1963) and marine vertebrates (Shewan, 1962). Lactic acid is a ubiquitous muscle constituent which accumulates as a result of muscular activity (White *et al.*, 1959, pp. 783–787) and post-mortem glycolysis (Tomlinson *et al.*, 1963). Furthermore, certain basic similarities in the chemical composition of many marine animals may account in part for the fact that *N. obsoletus* is not a "selective scavenger."

The reported experiments were confined to compounds found in fresh tissues. Products of bacterial decomposition may be equally stimulatory to this snail. Preliminary experiments revealed that the stimulatory capacity of a casein hydrolysate (prepared in sea water) increased considerably after standing at room temperature for 24 hours; bacterial activity was apparent because the solution became cloudy and developed a strong stench. However, it was considered that a greater contribution could be made by directing efforts toward the identification of endogenous tissue components which are themselves extremely effective response-inducers.

Of the compounds identified in shrimp extracts, only glycine (*ca.* 10^{-3} *M*) and lactate (*ca.* 5×10^{-4} *M*) possessed marked stimulatory capacities when tested individually. A third extract component, betaine (*ca.* 10^{-3} *M*), was mildly stimulatory. 2-Aminoethylphosphonic acid (*ca.* 5×10^{-4} *M*), N-acetylglucosamine (*ca.* 5×10^{-4} *M*), glycogen (*ca.* 0.06 mg./ml.), and pyruvate (*ca.* 5×10^{-4} *M*) possessed marked stimulatory capacities but were not identified in shrimp extracts. Henschel (1932) reported that glycine (*ca.* 10^{-3} *M*), lactate (*ca.* 10^{-2} *M*), and glycogen (*ca.* 0.06 mg./ml.) induced extensions of the proboscis by *Nassarius reticulatus*. It is noteworthy that these three compounds induce the same type of response in two related gastropods, one a North American species (*N. obsoletus*) and the other a European species (*N. reticulatus*). Trimethylamine (*ca.* 10^{-5} *M*), reported by Brown (1961) to be stimulatory to the gastropod, *Bullia laevissima*,

did not induce the PSR in *N. obsoletus* when tested at comparable concentrations.

The role of the osphradium in chemoreception by gastropods was first demonstrated by Copeland (1918) and later confirmed by Brown and Noble (1960). Bailey and Laverack (1963) found that stimulation of the osphradium of *Buccinum undatum* with *Mytilus* extracts, L-glutamic acid (*ca.* 10^{-3} *M*), and trimethylamine oxide (*ca.* 10^{-2} *M*) resulted in volleys of action potentials in the central nervous system. L-glutamic acid and trimethylamine oxide were not response-inducing in *N. obsoletus* at concentrations of 10^{-3} to 10^{-5} *M*. Electrophysiological techniques have been employed to study the sensitivity of chemoreceptors in marine arthropods to various compounds including glycine and betaine which induce the PSR in *N. obsoletus*. Barber (1961) reported that *Limulus polyphemus* possessed gnathobase receptors sensitive to glycine (*ca.* 10^{-3} *M*). Case and Gwilliam (1961) and Case (1964) found that dactyl receptors in several species of decapods are sensitive to a variety of amino acids (including glycine, *ca.* 5×10^{-2} *M*). Laverack (1963) showed that three species of decapods possess dactyl receptors sensitive to betaine (*ca.* 10^{-2} *M*) and trimethylamine oxide (*ca.* 10^{-2} *M*), but insensitive to glycine and several other amino acids. Levandowski and Hodgson (1965) found that the spiny lobster, *Panulirus argus,* possesses receptors on the antennules and dactyls which are sensitive to betaine, L-glutamic acid, trimethylamine, and trimethylamine oxide at concentrations of 10^{-3} *M*. These receptors were somewhat less sensitive to glycine (*ca.* 10^{-2} *M*). The possession of receptors sensitive to amino acids and/or other nitrogenous compounds of low molecular weight is characteristic of the marine arthropods and molluscs which have been studied.

Compounds with slight differences in molecular structure possessed markedly different stimulatory capacities in *N. obsoletus*. Of the 20 amino acids (19 L-amino acids and glycine) bioassayed over a concentration range of 10^{-3}–10^{-5} *M*, only glycine induced responses. Molecular modifications in the glycine (H_2NCH_2-COOH) moiety, as represented by the other amino acids ($H_2NCHRCOOH$), sarcosine (H_3CNHCH_2COOH), and glycylglycine ($H_2NCH_2CONHCH_2COOH$), resulted in a loss of stimulatory capacity at the concentrations at which glycine was effective (*ca.* 10^{-3} *M*).

Lactate ($CH_3CHOHCOO^-$) and pyruvate (CH_3COCOO^-) each induced a response of approximately 50% at 5×10^{-4} *M*; oxaloacetate ($^-OOCCH_2COCOO^-$) was less effective than either, while acetate (CH_3COO^-), beta-hydroxybutyrate ($CH_3CHOHCH_2COO^-$), malate ($^-OOCCH_2CHOHCOO^-$), and malonate (^-OO-CCH_2COO^-) were ineffective at comparable concentrations. Again slight changes in molecular configurations resulted in marked changes in stimulatory capacity.

2-Aminoethylphosphonic acid ($H_2NCH_2CH_2PO_3H_2$), reported by Quin (1965) to occur in at least three marine phyla, induced a response of 50% at 5×10^{-4} *M*; phosphoethanolamine ($H_2NCH_2CH_2OPO_3H_2$) was ineffective. The molecular difference in this case involved a C-P (a phosphonic acid) bond and a C-O-P (a phosphoryl ester) bond. N-acetylglucosamine induced a response of approximately 50% at 5×10^{-4} *M*; glucosamine was ineffective. The acetyl radical on the amino group of the former compound was the only molecular difference involved.

Since it was not the intent of this research to explore thoroughly the relative effectiveness of related compounds, the numbers and concentrations of tested compounds were limited. Nevertheless, the findings that closely related compounds

showed very different stimulatory capacities were suggestive of considerable receptor specificity. This aspect of chemoreception in *N. obsoletus* will be studied in more detail in the future.

Each solution prepared from an ion exchange or a paper chromatographic separation of a shrimp extract, which possessed a stimulatory capacity comparable to that of the total extract, was found to contain certain amino acids, *plus* betaine and lactate. Of these compounds, glycine, betaine, and lactate were each stimulatory when tested individually. However, the extracts themselves were effective at concentrations much lower than was attributable to the concentrations of glycine, betaine and lactate present (see Fig. 4). Moreover, a combination containing these three compounds in the same relative concentrations as they occurred in the extracts was no more effective than lactate alone. None of the nine amino acids, alanine, asparagine, aspartic acid, glutamic acid, glutamine, proline, serine, taurine, or threonine, was observed to be stimulatory when tested individually even at high concentrations (10^{-3} *M*). However, a combination containing these nine amino acids and glycine (Solution A), plus betaine and lactate, was considerably more effective than any of the individual compounds, or Solution A, or a combination containing only glycine, betaine, and lactate (see Fig. 4).

The effectiveness of the Solution A-betaine-lactate combination was due to an apparent synergistic effect. The fact that the stimulatory capacity of this combination was approximately 10–13 times as great as that of the glycine-betaine-lactate combination was not due to the presence of more total solute (*i.e.*, more organic molecules). The dose of Solution A-betaine-lactate which was sufficient to induce a response of approximately 50% contained only approximately 0.09 times as many micromoles of solute as the dose of glycine-betaine-lactate which was necessary to induce a comparable response. Hence the contributions of the components in the Solution A-betaine-lactate combination were not simply additive.

The *complete* stimulatory capacity of the shrimp extracts was not attained with the combinations of compounds which were tested. These combinations obviously lacked a component(s) which contributed to the effectiveness of the extracts themselves. Nevertheless, the results of these studies provide insight into factors which were difficult to consider in previous studies of chemoreception by aquatic invertebrates. The observation that certain compounds in an extract are stimulatory when tested individually does not justify the assumption that the stimulatory capacity of the extract resides entirely in these compounds. In this study, three compounds in a shrimp extract were found to be stimulatory when tested individually; however, these compounds were shown to be considerably less effective than the extract itself. Likewise, the observation that other compounds in an extract are individually non-stimulatory does not justify the assumption that such compounds make no contribution to the extract's stimulatory capacity. In this study, compounds in a shrimp extract which were individually non-stimulatory were found to contribute to the response-inducing capacity of a mixture of compounds.

Summary

1. A study was made of the compounds in shrimp extracts which induce the proboscis search reaction in *Nassarius obsoletus*.

2. Compounds identified in shrimp extracts were as follows: alanine, asparagine, aspartic acid, betaine, glutamic acid, glycine, histidine, homarine, inosine, isoleucine, leucine, lactic acid, lysine, methionine, phenlyalanine, proline, serine, taurine, threonine, trimethylamine oxide, tryptophan, tyrosine, urea, and valine. Carnitine was tentatively identified. The amino acids and lactic acid were determined quantitatively; betaine was determined semi-quantitatively.

3. Glycine (*ca.* 10^{-3} *M*) and lactate (*ca.* 5×10^{-4} *M*) were the only compounds identified in the extracts which possessed marked stimulatory capacities when tested individually; betaine (*ca.* 10^{-3} *M*) was mildly stimulatory. However, quantitative analyses of these compounds in shrimp extracts showed that they were present in insufficient concentrations to account for the responses observed with the dilutions of extract which were employed.

4. Elutions of extract components from paper chromatograms revealed that eluates from large portions of chromatograms were more effective than eluates from small portions. This implied that the stimulatory capacity of the total extract stemmed from the combined effect of a number of compounds.

5. The response-inducing capacity of a combination of twelve compounds identified in the extracts (glycine, lactate, betaine, alanine, asparagine, aspartic acid, glutamic acid, glutamine, proline, serine, taurine, and threonine) was greater than was attributable to the response-inducing capacities of the individual compounds. This combination of compounds possessed a stimulatory capacity which approached, though it did not attain, the stimulatory capacities of shrimp extracts. The effectiveness of this combination of compounds was not increased by the combined addition of histidine, isoleucine, leucine, lysine, methionine, phenylalanine, trimethylamine oxide, tryptophan, tyrosine, urea, and valine.

6. N-Acetylglucosamine (*ca.* 5×10^{-4} *M*), 2-aminoethylphosphonic acid (*ca.* 5×10^{-4} *M*), glycogen (*ca.* 0.06 mg./ml.), and pyruvate (*ca.* 5×10^{-4} *M*) also possessed marked response-inducing capacities in *N. obsoletus*. Ascorbate (*ca.* 10^{-4} *M*), citric acid (*ca.* 10^{-4} *M*), glucose (*ca.* 5×10^{-3} *M*), and oxaloacetic acid (*ca.* 10^{-4} *M*) were mildly stimulatory.

7. Compounds structurally related to N-acetylglucosamine, 2-aminoethylphosphonic acid, glycine, lactate, and pyruvate were either less effective or ineffective when tested at comparable concentrations. These findings suggest that considerable receptor specificity may exist.

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