EXPERIMENTS IN MARINE BIOCHEMISTRY:
I. HOMARINE METABOLISM
II. CHEMORECEPTION IN *Nassarius obsoletus*

By

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1974

ACKNOWLEDGEMENTS

The author would like to thank Dr. Samuel Gurin whose guidance, support, and tolerance proved invaluable throughout her graduate career. Furthermore, she would like to thank the other members of her committee: Drs. Bill Carr, Eugene Sander, and John Zoltewicz for their time and cooperation.

A very special thanks goes to Dr. Bill Carr for his honesty and interest and to Dr. Paul Cardeilhac for his strong shoulder, good ear, and sound advice. Finally, the author would like to thank Ms. Mary Smith, Ms. Peggy Osteen, and Mr. Dick Delanoy for their patience and help, without which it would have been impossible to persevere.

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Abstract of Dissertation Presented to the
Graduate Council of the University of Florida in
Partial Fulfillment of the Requirements for
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June, 1974

Chairman: Samuel Gurin
Major Department: Biochemistry

Homarine is endogenously synthesized by *Penaeus duorarum* in the free unbound form. The synthesis of homarine in *P. duorarum* was investigated by injecting shrimp with a series of C^{14}-labeled compounds. Following injection of d,l tryptophan (benzene ring-C^{14}(U)), no C^{14}-homarine was found, thereby showing that tryptophan is not a major precursor of homarine. Injection of acetic acid-2-C^{14} did result in the production of C^{14}-homarine. Previous investigators have shown that tryptophan is not labeled after the administration of C^{14}-acetate. The possibility that quinolinic acid undergoes decarboxylation and subsequent methylation to form homarine was then investigated by the injection of quinolinic-6-C^{14} acid. The homarine isolated had a

relatively high specific activity, suggesting that this compound is probably a major precursor of homarine. It seems likely, therefore, that 1) quinolinic acid is derived from more than one source in this species and 2) that it may be produced from an intermediate which can be synthesized from acetate. A condensation reaction between glyceraldehyde-3-phosphate and aspartic acid to form quinolinic acid has been described in higher plants and microorganisms. The incorporation of carbon 6 of quinolinic acid into homarine and the failure of incorporation of C^{14}-tryptophan suggested a study of aspartate. Although labeled aspartate is definitely converted to homarine, the radioactive yield was low. Whether this result was due to major dilution by endogenous free and bound aspartate is unknown. Finally, the injection of l-methionine (methyl-C^{14}) into P. duorarum resulted in C^{14}-homarine, providing evidence that S-adenosyl methionine probably contributes the N-methyl group of homarine.

Aqueous extracts of shrimp muscle were fractionated to determine the size and nature of the major stimulant(s) of the proboscis search reaction in Nassarius obsoletus. The size of the major stimulatory molecule(s) was estimated by ammonium sulfate precipitation, ultrafiltration through an Amicon UM 2 membrane, and Sephadex gel chromatography. The results obtained indicate that the active molecule(s) has a low molecular weight of approximately 1000. The activity of the major stimulant(s) was decreased 70% by aminopeptidase digestion, suggesting the involvement of a peptide. The

active molecule(s) was also shown to be soluble in 75% methanol and insoluble in acetone and chloroform. The 75% methanol-soluble material was subjected to electrophoresis at pH 4.8 and the anionic, cationic, and neutral fractions bioassayed. Of the 3 fractions, the anionic one was the only one with response-inducing activity. Upon spraying with ninhydrin, the anionic region revealed 4 ninhydrin-positive spots. Two of the spots were identified as aspartic and glutamic acids and shown to contain no activity. The other 2 spots did contain activity. Upon electrophoresis of the shrimp extract subsequent to aminopeptidase digestion, one of the unknown spots was eliminated and the other diminished in intensity. These results suggest that the active substance is a low molecular weight peptide that is anionic in character.

PART I

HOMARINE METABOLISM

CHAPTER I
INTRODUCTION

Homarine (1-methyl-2-pyridine carboxylic acid) was first reported in Crustacea by Hoppe-Seyler in 1933 (1). The intervening forty years have brought an elucidation of the pattern of homarine distribution; but they have yielded little enlightenment concerning its function, biosynthesis, or catabolism.

The distribution of homarine has been studied in a series of animals: basically, it has been found in most marine invertebrates below the Echinoderms and absent in terrestrial or freshwater species (2-5). For instance, Beers (2) estimated the concentration of homarine in the shrimp, Palaemonetes vulgaris, to be 0.60 - 1.19 mg/gm of wet weight; yet, no trace of homarine was found in freshwater crayfish by either Gasteiger et al. (3) or Leonard and MacDonald (5).

Gasteiger et al. (3) have also investigated the distribution of homarine by tissue in Loligo (squid), Homarus (lobster), and Limulus (king crab). In general, they found it had a wide distribution within the tissues of a given species with nerve and muscle tissues showing the highest concentrations (i.e., 10.3 mg/gm wet weight for the ventral

nerve cord of Limulus and 7.6 mg/gm for the cerebral ganglia of Loligo). Glandular tissue such as the hepatopancreas or gonads contained concentrations nearly as great, while the skin, mesentery, and stomach contained less. The blood and urine contained the least (i.e., 0.07 mg/gm wet weight for the blood of Limulus and 0.038 mg/gm for the blood of Loligo).

The area of homarine investigation generating the greatest interest and speculation has been that of homarine function. The presence of homarine in marine animals and its absence in corresponding freshwater animals has led to several investigations of its role in cellular osmoregulation. Levy (4) estimated homarine quantities in nerve cords of Limulus acclimatized to different salinities, but found no significant variations in homarine concentration when the external salinity was varied between 14.3 and 33.5 o/oo. Similarly, Dall (6) followed homarine concentrations in blood and whole animal samples from the crab Uca and shrimp Metapenaeus acclimatized to a range of salinities from 10 to 40 o/oo and found no evidence for a salinity effect. To date no direct role of homarine in osmoregulation has been demonstrated.

As a quaternary ammonium base concentrated in nerve and muscle tissue, homarine has also been suggested as playing a role in nerve function. Gasteiger et al. (3) investigated this possibility by perfusing lobster heart with homarine and its likely precursors and breakdown products.

They found that the threshold concentration of homarine required to alter the frequency and amplitude of the heartbeat was 10^7 times that of acetylcholine required; therefore, it was concluded to be improbable that homarine had a neurohumoral role. Likewise, Keyl et al. (7) found that homarine did not cause the contraction of the rectus abdominus muscle of the frog. Welsh and Prock (8) found homarine to have no observable paralyzing action on Uca pagilator and Kravitz et al. (9) found homarine to have a negligible neural inhibitory activity as compared to gamma aminobutyric acid (GABA) in the crustacean peripheral nervous system.

One other function for homarine has been suggested in the literature by Haake and Mantecon (10), who propose that homarine serves as a storage system for CO_2. They speculate that homarine is formed by the carboxylation of N-methylpyridinium ion forming a dipolar but neutral ion that could then be passed outside the cell, decarboxylated, and returned (by active transport) back into the cells as N-methylpyridinium. This theory has never been investigated, but appears as an unlikely solution for it would represent an energetically expensive means of CO_2 release.

In 1971, Dall (6) addressed the question of homarine origin in shrimp. He proposed that homarine is made by the methylation of picolinic acid derived by the breakdown of tryptophan. Dall injected each of three Metapenaeus with 10 μCi of C^{14}-tryptophan, homogenizing them (two after 24 hr and one after 72 hr) in methanol. The methanol extract was

dried, extracted with water, and chromatographed on thin layer plates. Since radioactivity was found in the UV absorbing spot corresponding to synthetic homarine, C^{14}-homarine was assumed to have been synthesized from C^{14}-tryptophan. However, it is doubtful that this procedure purified homarine from all traces of tryptophan, thus casting serious doubt on the results and their interpretation.

The present work takes another look at the question of homarine origin. A practical purification scheme for microquantities of homarine is described and data indicating that indeed homarine is synthesized endogenously are presented.

CHAPTER II
MATERIALS AND METHODS

The Maintenance and Injection of Penaeus duorarum

The selection of the shrimp, Penaeus duorarum, for these experiments was based on several factors: 1) they are readily accessible, 2) they can be maintained in the laboratory for an indefinite period, 3) they contain workable quantities of homarine, and 4) they are easy to inject.

Penaeus duorarum utilized in this study were generally collected from the Cedar Key area and acclimatized within the laboratory for at least 24 hr. They were housed in glass aquaria at room temperature with constant aeration and filtration of the sea water. They could be maintained for several months under these conditions on a diet of Biorell fish food (Sternco).

High survival rates were observed when shrimp were injected either intravenously or intramuscularly. Intravenous injections were made through the articular membrane of the fifth abdominal segment just to the left of the mid-dorsal line, while intramuscular injections were made into the ventral portion of the first abdominal segment. See Figures 1 and 2.

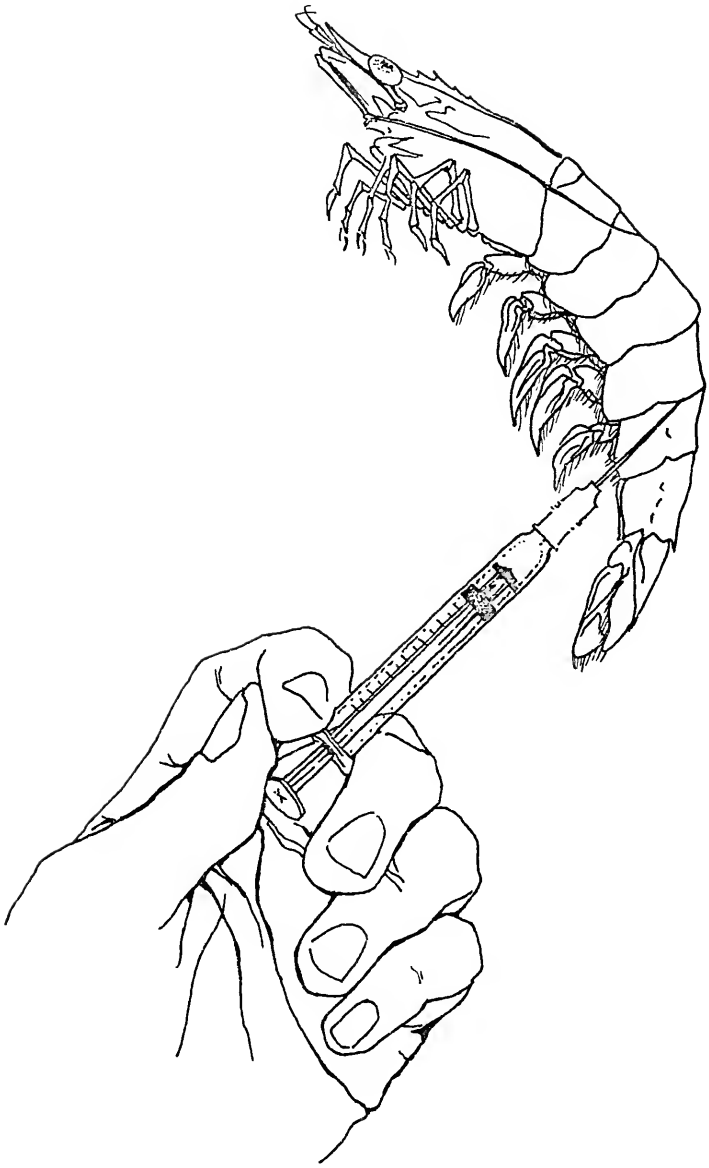


Figure 1. Administration site of intravenous injections in Penaeus duorarum.

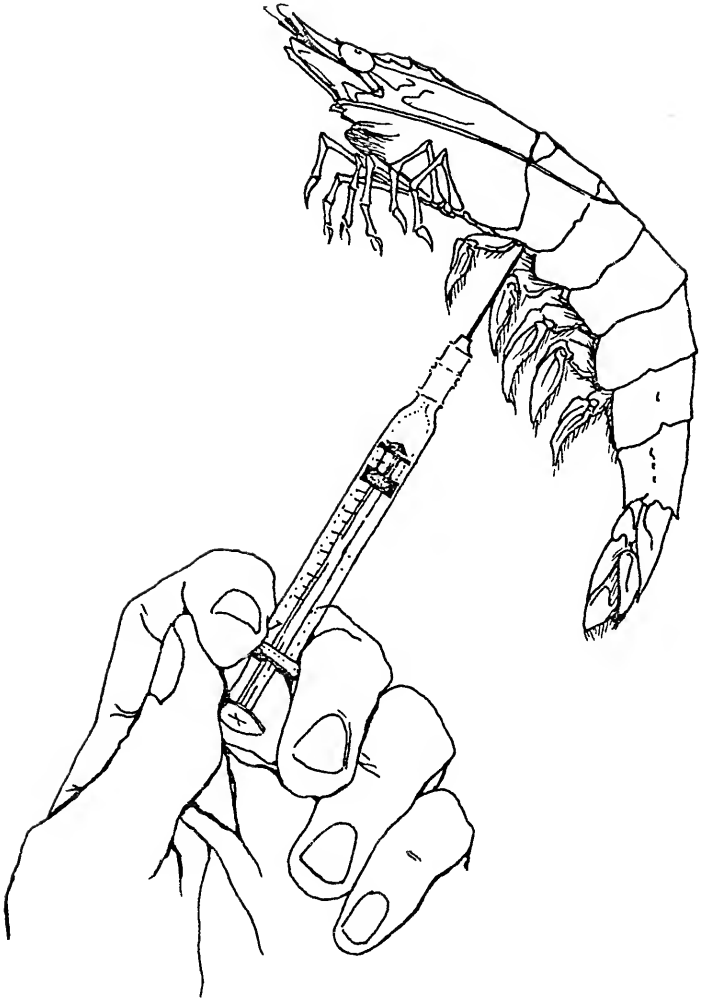


Figure 2. Administration site of intramuscular injections in Penaeus duorarum.

Chromatographic Procedures Utilized in Homarine Fractionation

Of the various chromatographic procedures available to the biochemist, three were selected for use in this study. They were exchange chromatography (anion and cation), thin-layer chromatography, and Sephadex gel chromatography.

Anion exchange chromatography. At a pH of 10.9 most of the dipolar ions present in shrimp extract, including the amino acids, are negatively charged and thus retained by an anion exchange resin. Homarine, however, was observed to pass unretarded through a strong anion exchange column at pH 10.9. This fact was employed in the purification of homarine from an alcoholic shrimp extract by chromatographing the shrimp extract on a 2.5 x 21 cm column of AG1-x8 resin (OH^- form, 200-400 mesh) that was equilibrated and eluted with 0.5% NH_4OH (pH 10.9). Homarine hydrogen sulfate and other related compounds were chromatographed as described in order to ascertain which ones were and were not retained by the AG1-x8 anion exchange column. The results given in Table 1 show that the amino acids, glycine and tryptophan, and the pyridine carboxylic acids, picolinic, nicotinic, and quinolinic were all retained by the column; whereas the n-methyl pyridine carboxylic acids, homarine and trigonelline passed unretarded through the column.

Cation exchange chromatography. Initial experiments with a strong cation exchange resin showed homarine to be firmly bound to the resin and eluted only after the passage

TABLE 1

THE RETENTION OF STRUCTURALLY RELATED COMPOUNDS
ON AN AG1-x8 ANION EXCHANGE COLUMN

Compound	Retained	Not Retained
Homarine	-	+
Trigonelline	-	+
Quinolinic acid	+	-
Picolinic acid	+	-
Nicotinic acid	+	-
Glycine	+	-
Tryptophan	+	-

of approximately 15 bed volumes of 0.1N HCl. A 1.5 x 28 cm column of AG50W-x8 resin (H^+ form, 200-400 mesh) was poured and washed with a minimum of five bed volumes of 2N HCl. The column was then washed thoroughly with water until the effluent pH was raised to 6. The sample to be chromatographed was applied to the column and the column washed with 200-300 ml of water and eluted with 0.1N HCl. The eluate was collected in 6 min fractions of approximately 11 ml each. The UV 274 absorbance (the λmax for homarine) of each fraction was measured and plotted against the eluate volume.

A trial run was made with a mixture of homarine hydrogen sulfate and trigonelline (1-methyl-3-pyridine carboxylic acid). Trigonelline came off the column during the water wash whereas homarine was eluted only after the passage of 800-1000 ml of 0.1N HCl as shown in Figure 3.

Sephadex gel chromatography. Dall (6) has reported that the homarine and tissue proteins found in Metapenaeus blood are inseparable by Sephadex G-10 chromatography. In an effort to clarify this observation, Blue Dextran (mw 2 x 10^6) was mixed first with a sample of homarine hydrogen sulfate and then with a sample of homarine isolated from shrimp. Each mixture was chromatographed on a Sephadex G-10 column (1 x 53 cm) equilibrated and eluted with phosphate buffer (0.01 M, pH 7.5). The eluate was collected in 4 ml fractions and the UV 274 absorbance measured and plotted against the eluate volume. The results shown in Figures 4 and 5 show that the homarine isolated

Figure 3. The elution of homarine from an AG50W-x8 (cation exchange column) with the passage of 0.1N HCl. The eluate was collected at 6 min intervals and the eluate volume plotted against the recorded UV 274 absorbance (the λ max for homarine). Homarine was eluted from the column only after the passage of 800-1000 ml of 0.1N HCl.

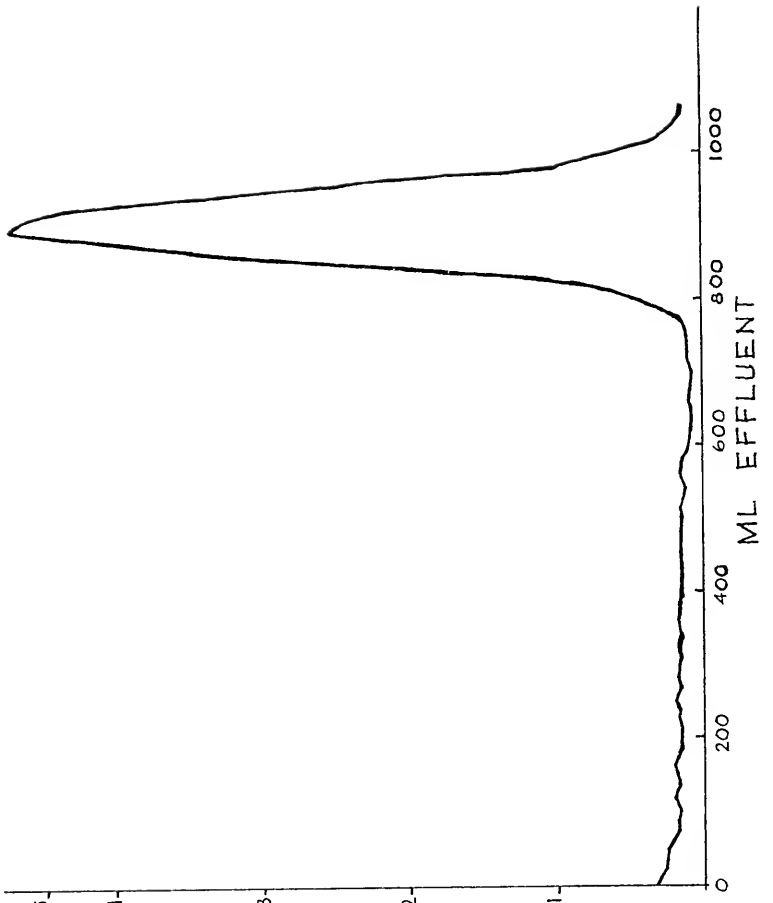

UV ABSORBANCE 274 mμ
.45
.4
.3
.2
.1
0
200
400
600
800
1000
ML EFFLUENT

Figure 4. Separation of synthetic homarine hydrogen sulfate from Blue Dextran (mw 2 x 10^6) on a Sephadex G-10 column (1 x 53 cm). Peak 1 (18-24 ml) represents the elution of Blue Dextran from the column, while Peak 2 (27-34 ml) represents the elution of the homarine hydrogen sulfate.

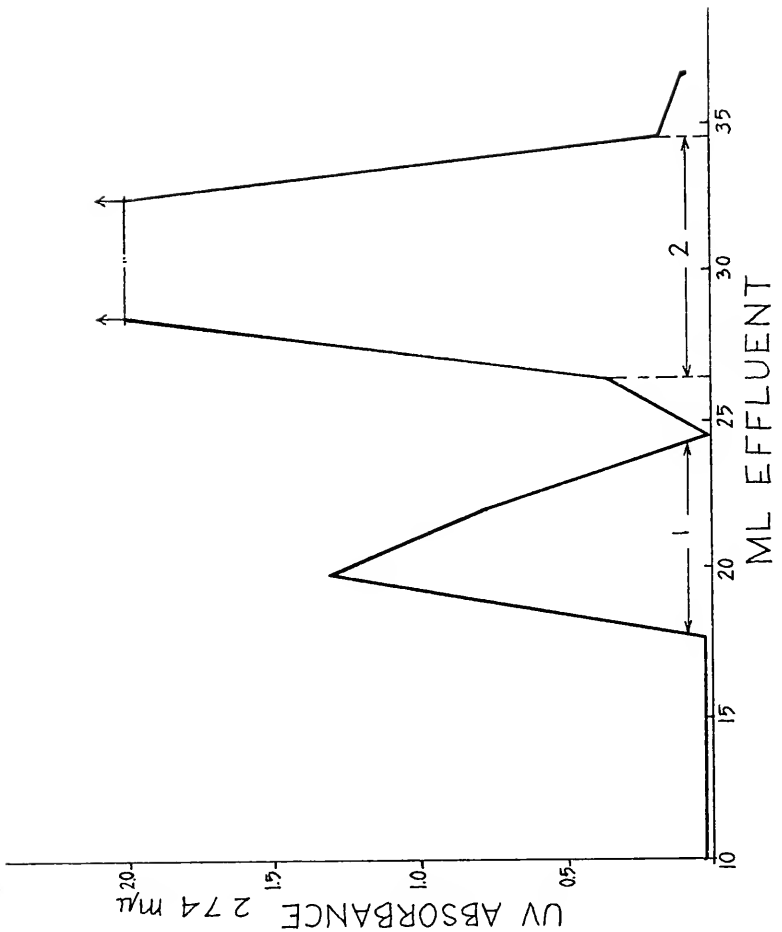
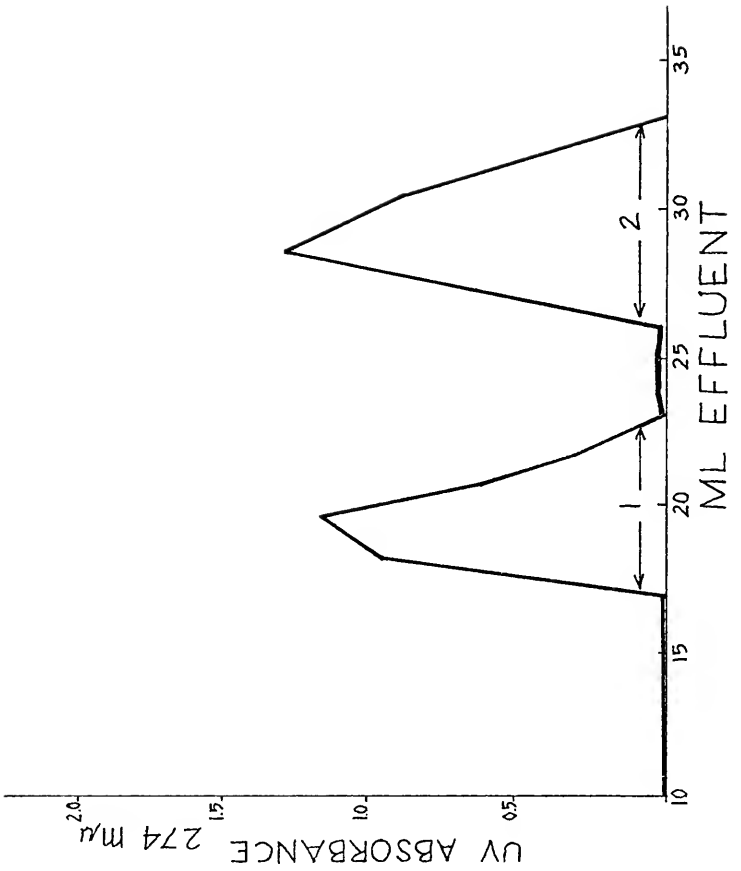

UV ABSORBANCE 274 mμ
0.5
1.0
1.5
2.0
10
15
20
25
30
35
ML EFFLUENT
1
2

Figure 5. Separation of homarine isolated from shrimp extract from Blue Dextran on a Sephadex G-10 column (1 x 53 cm). Peak 1 (17-23 ml) represents the elution of Blue Dextran from the column, while Peak 2 (26-33 ml) represents the elution of homarine isolated from shrimp.


UV ABSORBANCE 274 mμ
2.0
1.5
1.0
0.5
10
15
20
25
30
35
ML EFFLUENT
1
2

from shrimp separated from the Blue Dextran in a manner analogous to that of the synthetic homarine hydrogen sulfate.

Thin layer chromatography. Homarine hydrogen sulfate and a series of related compounds were run on microcrystalline cellulose plates (250μ) in a variety of solvent systems. Two solvent systems, 60:20:20 butanol:acetic acid:water and 90:5:5 methanol:acetic acid:water, were selected and used throughout this study. The Rf values of homarine and related compounds in these two solvent systems are listed in Table 2. In the acidic butanol system homarine had an Rf value of 0.41 and was well separated from nicotinic and picolinic acids but not from trigonelline. In the acidic methanol system homarine had an Rf value of 0.66 and was well separated from nicotinic and picolinic acids and trigonelline.

Chromatographic examination of isolated homarine fractions, using two different solvent systems, revealed only one UV absorbing spot in each case. The UV absorbing spot, which had the same Rf values as the synthetic homarine hydrogen sulfate, gave a yellow color when sprayed with alkaline α-naphthol (equal volumes of 5N NaOH and 1% α-naphthol in ethanol) as described by Leonard and MacDonald (5). No ninhydrin-reacting compounds were detected on these chromatograms.

TABLE 2

Rf VALUES OF HOMARINE AND RELATED COMPOUNDS
RUN ON MICROCRYSTALLINE CELLULOSE PLATES

Solvent System	Compound	Rf
I[a]	Homarine	0.41
	Trigonelline	0.42
	Picolinic acid	0.57
	Nicotinic acid	0.73
II[b]	Homarine	0.66
	Trigonelline	0.54
	Picolinic acid	0.78
	Nicotinic acid	0.83

[a]Solvent System I: 60:20:20 butanol:acetic acid:water

[b]Solvent System II: 95:5:5 methanol:acetic acid:water

Precipitation of Homarine with Phosphotungstic Acid

Homarine phosphotungstate, a relatively insoluble salt, was precipitated cold from an acidic homarine solution (approximately 1N H_2SO_4) with the addition of 10% phosphotungstic acid. This salt, following a wash with an acid phosphotungstate solution and solvation in 0.5N NaOH, was reprecipitated by lowering the pH to 1 in the presence of phosphotungstic acid. The resulting precipitate was washed and dissolved as before.

Removal of the phosphotungstate was accomplished by the addition of 10% BaOH to precipitate barium phosphotungstate. Excess barium, provided to ensure complete phosphotungstate removal, was in turn removed as barium sulfate by the addition of 2N H_2SO_4 to a pH of 1 leaving a solution of homarine hydrogen sulfate.

Isolation of Homarine from Penaeus duorarum Extracts

The primary prerequisite of this project was to perfect a practical purification scheme for the isolation of milligram quantities of homarine from extracts of shrimp muscle.

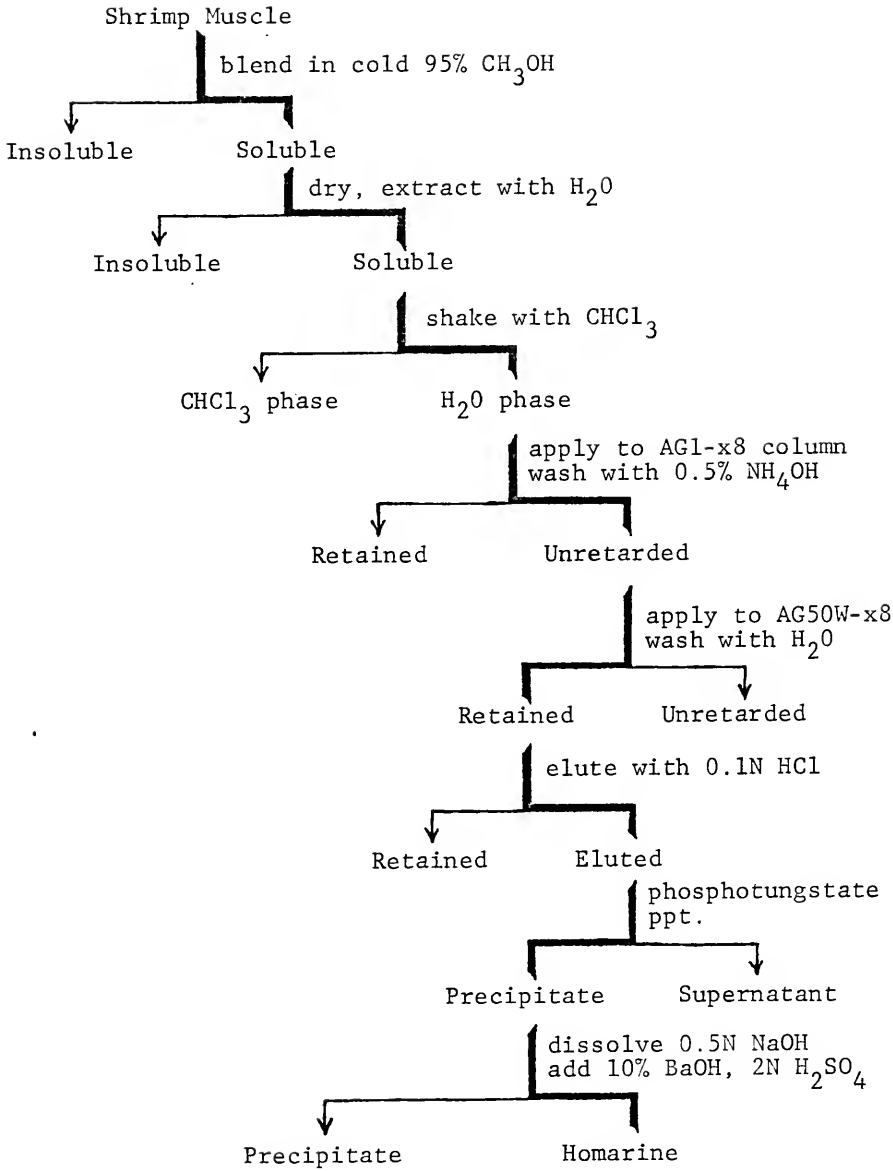
Fresh shrimp muscle (5-20 gm) was blended 3 times in 100 ml of cold 95% ethanol and centrifuged at 10,000 rpm for 10 min. The residue left after evaporating the combined supernatants was dissolved in 15 ml of water, shaken with 2 ml of chloroform, and centrifuged. The aqueous phase was

again shaken with 2 ml of chloroform and centrifuged. The chloroform phases were combined and washed with 5 ml of water. The two aqueous phases were then combined and evaporated to dryness in a rotary evaporator.

The resulting residue was dissolved in 5 ml of water and passed through an AG1-x8 column at pH 10.9. The 0.5% NH_4OH (pH 10.9) eluate was neutralized with hydrochloric acid, concentrated to approximately 5 ml, and chromatographed on an AG50W-x8 column. The homarine-containing fraction was eluted with the passage of 800-1000 ml of 0.1N HCl and detected by its UV absorbance as seen in Figure 3. The eluted homarine fraction was reduced in volume to 1-3 ml and the homarine precipitated with phosphotungstic acid as previously described. Figure 6 gives a flow chart of the homarine isolation procedure as applied throughout this study.

The UV spectra of the isolated homarine hydrogen sulfate appeared identical to that of synthetic homarine hydrogen sulfate where the λ_{max} was 274 nm and the λ_{min} was 243 nm at pH 1. The extinction coefficient (5,11) found for synthetic homarine (6,200) was thus used to calculate the concentration of homarine present in isolated homarine fractions. The isolated and synthetic compounds also gave the same Rf values when run on thin layer plates in two solvent systems.

Figure 6. Flow sheet illustrating the fractionation procedures utilized in the isolation of homarine from shrimp extracts.


Shrimp Muscle
blend in cold 95% CH_3OH
Insoluble
Soluble
dry, extract with H_2O
Insoluble
Soluble
shake with $CHCl_3$
$CHCl_3$ phase
H_2O phase
apply to AG1-x8 column
wash with 0.5% NH_4OH
Retained
Unretarded
apply to AG50W-x8
wash with H_2O
Retained
Unretarded
elute with 0.1N HCl
Retained
Eluted
phosphotungstate
ppt.
Precipitate
Supernatant
dissolve 0.5N NaOH
add 10% BaOH, 2N H_2SO_4
Precipitate
Homarine

Procedures Used in the Treatment of Radioactive Homarine Fractions

Isolated homarine fractions ranging from 0.1-0.5 ml in volume were counted in a Beckman LS 230 liquid scintillation counter using a toluene based cocktail with 10% v/v of BBS-3 and 0.8% wt/v of TLA fluor (12). Background counts for 0.1-0.5 ml of synthetic homarine hydrogen sulfate (1 mg/ml) were determined to be 31 ± 2 cpm for the C^{14} ISO-SET, with a calculated counting efficiency of 91%. All samples were counted for a minimum of 5 10-min counts and the average cpm calculated.

Aliquots of active homarine fractions (fractions having greater than 10 cpm above background) were streaked on 500μ microcrystalline cellulose plates and run in two solvent systems. The homarine band was scraped from the plates and extracted with water. Estimated specific activities of the chromatographed homarine samples were calculated and compared to that of the original homarine fraction.

CHAPTER III
RESULTS

Crayfish Feeding Experiment

The question of whether homarine is synthesized by shrimp or merely ingested and stored was originally approached by feeding shrimp a non-homarine diet while monitoring their homarine content.

Twelve shrimp were maintained on a diet devoid of homarine by feeding them frozen crayfish (*Cambarus sp.*) for 21 days. At 7-day intervals, 3 shrimp were killed and their homarine content estimated. The values obtained for the 3 samples from each group were averaged and the averages compared. As seen in Table 3, greater variation was observed in the homarine concentrations of the shrimp within any one group of samples than between the average concentrations of the different groups.

Since the homarine content did not significantly decrease over this 3-week period, it was considered quite probable that homarine was being synthesized by the shrimp rather than being obtained from the diet.

TABLE 3

HOMARINE CONCENTRATIONS FOUND
IN CRAYFISH-FED SHRIMP

No. of Days on Crayfish Diet	Homarine Concentration (mg/gm) Shrimp 1	Shrimp 2	Shrimp 3	Average
0	0.98	0.59	0.50	0.69
7	1.11	0.37	0.55	0.68
14	1.16	0.79	0.53	0.83
21	0.51	0.76	0.30	0.51

The State of Homarine in Shrimp: Free or Bound

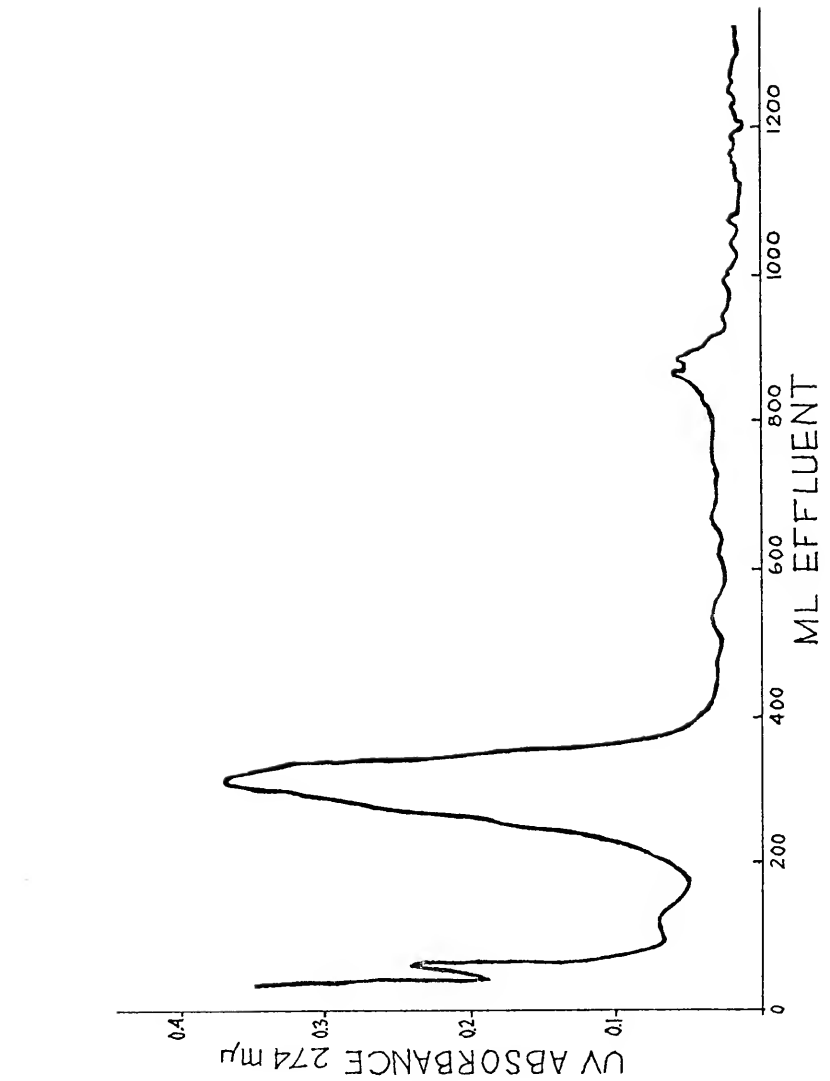
Encouraged by the results of the crayfish feeding experiment, a series of injection studies with C^{14}-labeled precursors were planned. However, before any labeling experiments could be done, it was necessary to determine the state of homarine (free or bound) in the shrimp.

Dall (6) has suggested that homarine appears bound to a small peptide in the shrimp, Metapenaeus. This question was investigated in Penaeus duorarum by considering each of 3 possibilities: 1) that homarine is bound to an alcohol insoluble peptide or protein, 2) that homarine is bound to a small alcohol soluble peptide, and 3) that homarine appears in the free state.

Fresh shrimp muscle was blended 3 times in cold 95% ethanol and centrifuged at 10,000 rpm for 10 min. The ethanol-insoluble precipitate was hydrolyzed in 6N HCl at 100°C for 28 hrs. The hydrolyzate was then chromatographed on an AG50W-x8 cation exchange column. The eluate was collected in 6 min fractions and the UV 274 absorbance measured and plotted against the eluate volume. The UV 274 absorbance shown in Figure 7 within the region of homarine elution (800-1000 ml) represents less than 1% of the homarine subsequently recovered from the alcohol-soluble fraction.

The ethanol-soluble fraction of the shrimp extract was then evaporated to dryness. The residue was thoroughly

Figure 7. Fractionation of hydrolyzed alcohol-insoluble material from shrimp muscle on an AG50W-x8 cation exchange column. The eluate was collected in 6 min fractions and its UV 274 absorbance plotted against the eluate volume. The UV 274 absorbance appearing within the region of homarine elution represents less than 1% of the homarine found in the alcohol-soluble fraction.


UV ABSORBANCE 274 mμ
0.4
0.3
0.2
0.1
0
200
400
600
800
1000
1200
ML EFFLUENT

extracted with 10 ml of water, of which 1 ml aliquots were chromatographed on an AG50W-x8 column before and after acid hydrolysis. Theoretically, free and bound homarine should have different chromatographic properties, such that if a homarine-peptide were hydrolyzed, the eluted homarine peak would be increased in proportion to the quantity of bound homarine present. Yet a comparison of Figures 8 and 9 shows that the homarine peak is not increased upon hydrolysis. The amount of homarine eluted from the column was 0.38 mg from the unhydrolyzed fraction and 0.34 mg from the hydrolyzed fraction.

Thin layer chromatograms of homarine isolated from shrimp were also run before and after acid hydrolysis. Both chromatograms had a single UV absorbing spot and neither contained any ninhydrin-reactive material. Furthermore, synthetic and isolated homarine fractions exhibited identical chromatographic properties on a Sephadex G-10 column as seen in Figures 4 and 5.

The evidence presented here strongly suggests that most, if not all, of the homarine exists in Penaeus duorarum in its free molecular form.

The Injection of C^{14}-Labeled Compounds

Additional evidence for the synthesis of homarine by shrimp was obtained by injecting C^{14}-labeled compounds into the shrimp either intravenously or intramuscularly with the

Figure 8. Chromatographic separation of the ethanol-soluble material in shrimp homogenates. The homarine-containing fraction is labeled as peak 1 and contains 0.38 mg of homarine.

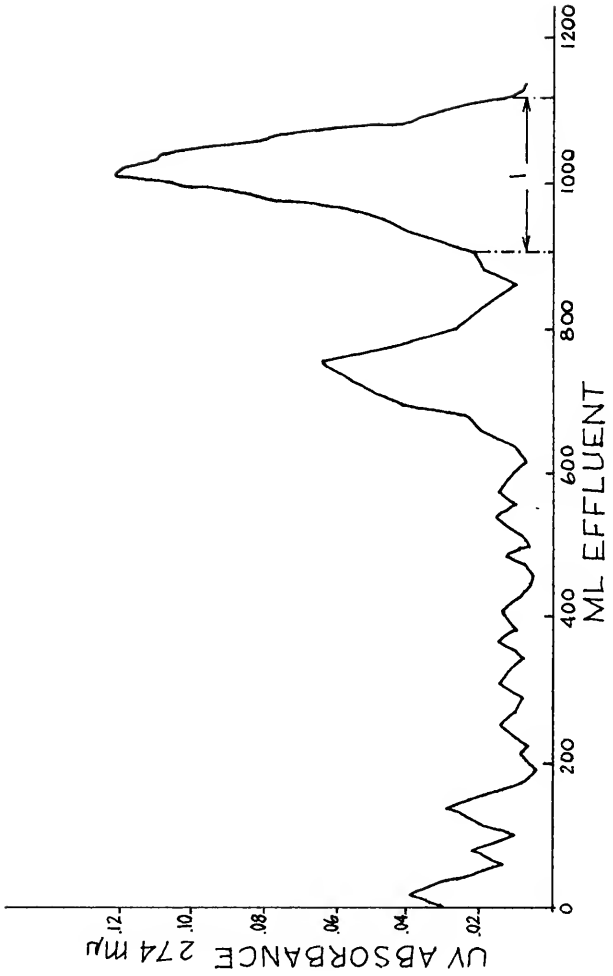
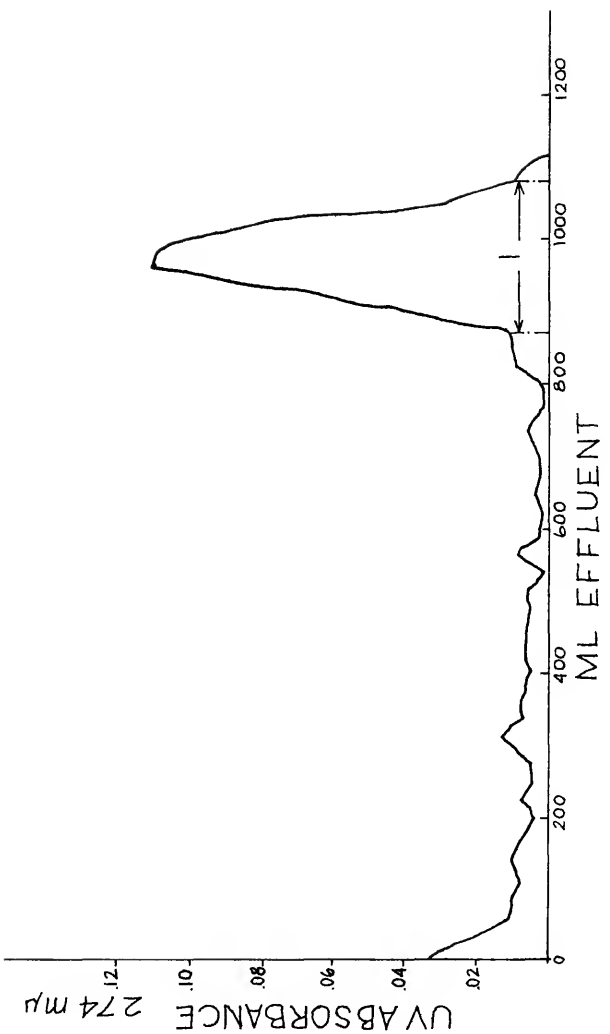

UV ABSORBANCE 274 mμ
.12
.10
.08
.06
.04
.02
0
200
400
600
800
1000
1200
ML EFFLUENT
1

Figure 9. Chromatographic separation of the hydrolyzed ethanol-soluble material. The homarine-containing fraction is labeled as peak 1 and contains 0.34 mg of homarine.


UV ABSORBANCE 274 mμ
.12
.10
.08
.06
.04
.02
0
200
400
600
800
1000
1200
ML EFFLUENT

subsequent isolation of homarine. Whenever C^{14}-homarine was isolated, it was considered to have been synthesized from the injected C^{14}-labeled compound. When C^{14}-homarine could not be isolated after the injection of a C^{14}-labeled compound, that compound was considered to be of little importance in the derivation of homarine.

C^{14}-tryptophan. The close chemical relationship of homarine to nicotinic acid and its N-methyl derivative, trigonelline, has led to suggestions that homarine is a product of tryptophan catabolism (6). Five μCi of d,l tryptophan (benzene ring-C^{14}(U)) were injected into the vascular system of shrimp and a crude homarine fraction isolated 23 hrs later by thin layer chromatography. When this sample of homarine was rechromatographed with cold, carrier tryptophan the calculated specific activity was only 1/4 that of its previous value. Thus it was apparent that the homarine fraction being counted was not pure homarine; therefore the experiment was repeated after the development of an improved purification scheme for homarine.

This time ten μCi of d,l tryptophan (benzene ring-C^{14}(U)) were injected into three shrimp. Two shrimp were injected with 2μCi each and killed after 6 hrs. The third shrimp was given two 3μCi-injections 12 hrs apart and was killed 12 hrs after the second injection. Alcoholic extracts of the 3 shrimp were combined and the homarine isolated. Although 7 mg of homarine were obtained from the C^{14}-tryptophan injected shrimp, the isolated homarine fraction contained no

counts above background. See Table 4.

Isolated and synthetic homarine fractions gave the same Rf values in two solvent systems and had identical UV spectra.

C^{14}-acetate. Having ruled out tryptophan as a major precursor utilized by the shrimp in homarine synthesis, C^{14}-labeled acetate was employed in an effort to obtain C^{14}-homarine and thus provide additional support for the thesis of homarine synthesis by the shrimp.

Two 62.5μCi-injections of acetic-2-C^{14} acid were given 12 hrs apart. After twelve additional hrs the shrimp was killed and its homarine isolated. The 4.5 mg of homarine isolated were found to contain a total of 2220 dpm. Aliquots of this homarine fraction run in the acidic butanol and acidic methanol solvent systems retained their activity as seen in Table 4. The estimated specific activities of the homarine run in the acidic butanol and the acidic methanol solvent systems were 85.4% and 98.6% of the specific activity of the original fraction. The fact that C^{14}-homarine was isolated from shrimp injected with C^{14}-acetate provided additional support for two points: 1) that shrimp do in fact synthesize homarine, and 2) that tryptophan is not a direct precursor of homarine, since tryptophan is not labeled with the injection of C^{14}-acetate (13).

C^{14}-quinolinic acid. The possibility that quinolinic acid undergoes decarboxylation and subsequent methylation in

TABLE 4

THE ACTIVITY OF HOMARINE FRACTIONS ISOLATED FROM SHRIMP INJECTED WITH LABELED PRECURSORS

Labeled Compound	μCi	Total dpm Recovered	Homarine Recovered (mg)	Spec. Act.[a] (dpm/mg)	Spec. Act. of Chromatographed Homarine Fractions (dpm/mg) I[b]	II[c]
d,l-Tryptophan (benzene ring-C^{14}(U))	10	0	7.1	------	------	------
Acetic-2-C^{14} acid	125	2220	4.5	493	421	486
Quinolinic-6-C^{14} acid	15	879	0.78	1127	1409	1323
l-Aspartic acid-C^{14}(U)	25	121	16.9	7.1	7.1	6.8
l-Methionine (methyl-C^{14})	100	557	3.2	174	151	140

[a]Spec. Act.: The estimated specific activity of isolated homarine fractions.

[b]I: The estimated specific activity of the homarine fraction after being chromatographed on cellulose plates in a 60:20:20 butanol:acetic acid:water solvent system.

[c]II: The estimated specific activity of the homarine fraction after being chromatographed on cellulose plates in a 95:5:5 methanol:acetic acid:water solvent system.

shrimp to form homarine was investigated by injecting 15μCi of quinolinic-6-C^{14} acid into Penaeus duorarum. Twelve hrs later the shrimp was homogenized. The isolated homarine fraction had an estimated specific activity of 1127 dpm/min.

Subsequent chromatography in the acidic butanol and acidic methanol solvent systems did not decrease the activity of the homarine fraction as seen by the estimated specific activities of these fractions listed in Table 4. The fact that carbon 6 of quinolinic acid is incorporated into homarine supports the proposed pathway shown in Figure 10. Furthermore, the high specific activity of the isolated homarine fraction suggests that quinolinic acid is an important precursor of homarine.

C^{14}-aspartate. Leete (14) and others (15, 16) have proposed that quinolinic acid is formed by a condensation reaction between glyceraldehyde-3-phosphate and aspartic acid in higher plants and certain microorganisms. See Figure 11. The incorporation of carbon 6 of quinolinic acid into homarine and the lack of incorporation with C^{14}-tryptophan made this pathway an attractive possibility.

One shrimp was injected with 25μCi of l-aspartic acid-C^{14}(U) and killed 9 hrs later. The homarine isolated from the shrimp had only 121 dpm in the 16.9 mg isolated, or an estimated specific activity of 7 dpm/mg. The homarine fraction retained its activity after chromatographing it in two different solvent systems as seen in Table 4. Apparently C^{14}-aspartate can contribute carbon atoms to homarine, yet not as readily as C^{14}-acetate.

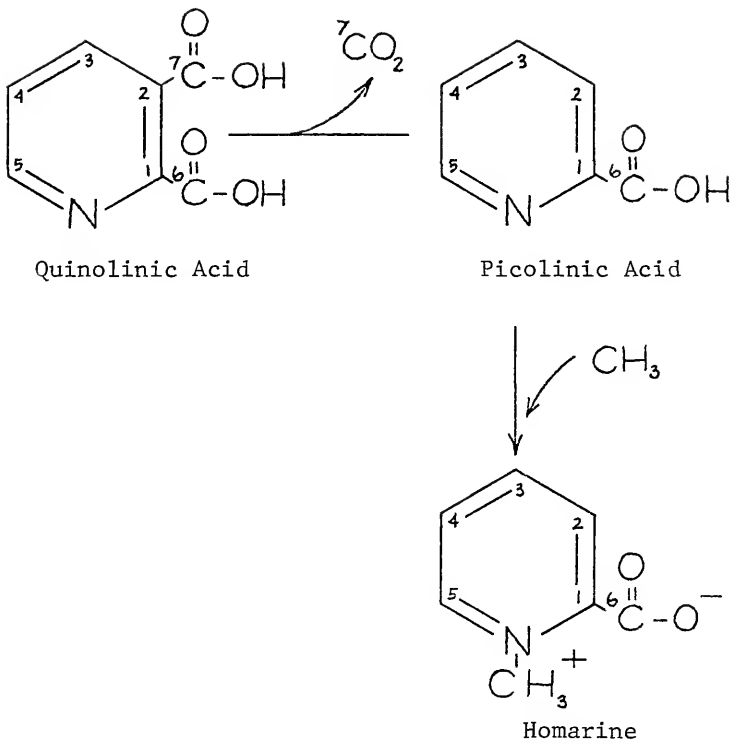


Figure 10. A proposed pathway for the incorporation of carbon 6 of quinolinic acid into the homarine molecule.

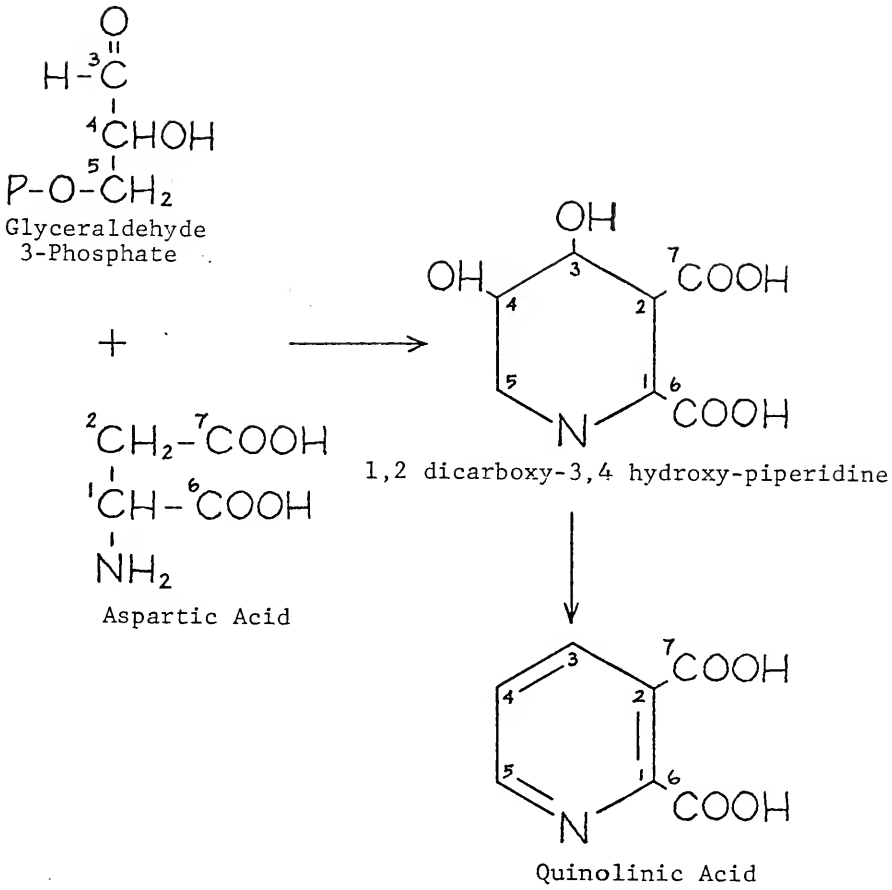


Figure 11. Biogenetic scheme for the formation of quinolinic acid in higher plants and some microorganisms as proposed by Leete (14) and others (15, 16).

C^{14}-methionine. In order to determine whether the methyl group of homarine is derived from methionine, two shrimp were given intramuscular injections of 50μCi of l-methionine (methyl-C^{14}). The shrimp were homogenized after 12 hrs and their homarine extracted. The 3.2 mg of homarine isolated contained a total of 557 dpm. Aliquots of this homarine fraction run in the acidic butanol and acidic methanol solvent systems retained their activity as seen in Table 4.

The fact that the C^{14} from the methyl group of methionine was incorporated into homarine provides strong support for the suggestion that S-adenosyl-methionine contributes the N-methyl group of homarine.

CHAPTER IV
DISCUSSION

Evidence has been presented which demonstrates that homarine is endogenously synthesized by Penaeus duorarum and that most, if not all, of it exists unbound as free homarine.

Tryptophan is known to give rise to nicotinic acid, which is closely related to picolinic acid, via a quinolinic acid pathway. Thus, it has been tempting to assume that homarine is produced by essentially the same pathway. However, the results given indicate that tryptophan is not an important precursor of homarine, for not only did injections of C^{14}-tryptophan yield inactive homarine, but labeled acetate was converted to labeled homarine; and Cowey and Forster (13) have shown that tryptophan is not labeled after the administration of C^{14}-acetate.

Results obtained by the administration of radioactive quinolinic acid suggest that this compound is probably a major precursor of homarine. It seems likely, therefore, that 1)quinolinic acid is derived from more than one source in this species and 2) that it may be produced from an intermediate which can be synthesized from acetate.

There are very few metabolic pathways known to give rise to quinolinic acid. Mention has been made (14-16) of

a condensation between glyceraldehyde-3-phosphate and aspartate which gives rise to quinolinic acid. Although labeled aspartate is definitely converted to homarine, the radioactive yield was low. Whether this result is due to major dilution by endogenous free and bound aspartate is not clear.

Since labeled acetate appears to be readily incorporated into homarine, it will be of interest to test other metabolites that may be derived from acetate: pyruvate, short chain fatty acids, members of the tricarboxylic acid cycle and the non-essential amino acids. Although it would appear to be highly unlikely, there is always the possibility that acetate may condense with a nitrogen-containing metabolite derived from one of the essential amino acids.

Finally, these experiments indicate that homarine is derived by decarboxylation of quinolinic acid followed by subsequent methylation of the ring nitrogen. It is probable that the latter reaction occurs via S-adenosyl methionine.

PART II

CHEMORECEPTION IN *Nassarius obsoletus*

CHAPTER V
INTRODUCTION

Chemical attractants which may act over long distances to orient an animal toward the apparent source of those chemicals are of widespread importance in food localization (17). For example, turkey vultures are attracted and will orient to ethyl mercaptan dispersed in the air by a fan and it has long been known that sharks are attracted to very low concentrations of vertebrate blood. Chemical attractants that act over long distances must be freely diffusable in the environment of the animal that is to be attracted (i.e., attractants for terrestrial animals must be volatile and those for aquatic animals water soluble).

Although some basic work has been done on chemoreception in marine invertebrates in general and gastropods in particular, any understanding of this phenomenon at the molecular level awaits the identification of the compounds involved (17, 18, 19). The majority of chemoreception studies have been oriented along three major lines of investigation: 1) proof that observed responses in certain animals are chemically induced, 2) investigations of the chemical nature of attractants, and 3) tests of a spectrum

of known compounds for their stimulatory activity. Then in 1967, Carr made a significant attempt to account for the responses of the marine mud snail Nassarius obsoletus to shrimp extracts, both in terms of the compounds present and their relative concentrations (20,21).

Nassarius obsoletus is particularly suitable for chemoreception studies, as it displays a stereotyped response (i.e., extending its proboscis) which is convenient for measuring the effectiveness of stimulatory substances. Using the proboscis search reaction as described by Carr (20), Gurin and Carr (22) were able to show that the stimulation induced by human serum and by oyster mantle fluid was attributable primarily to very low concentrations of specific proteins. In serum the major stimulant was highly purified serum albumin (ca. 10^{-9} M), whereas in oyster fluid the major stimulant proved to be a homogenous glycoprotein (ca. 10^{-10}). This glycoprotein accounted for more than 90% of the stimulatory activity of the oyster mantle fluid. This was the first time that an attractant had been isolated from an animal fluid and shown to account for essentially all of the activity of the natural fluid.

Carr et al. (23) screened biological fluids and extracts from eight species of marine animals to determine the nature of the principal inducers of stimulatory activity in Nassarius obsoletus. The major response inducers from the scallop, clam, blue crab, sea urchin, and three fishes proved to be macromolecules that were ammonium

sulfate precipitable, non-dialyzable, and retained by ultrafiltration using an Amicon UM 2 membrane. In contrast, analyses of various fractions obtained from shrimp extracts show that their major response inducers are low molecular weight substances which are dialyzable and are included in the bead matrices of Sephadex G-10 columns which will exclude globular molecules with molecular weights of 700 or more.

A variety of low molecular weight substances, such as amino acids, betaines, and amines, identified in shrimp extracts have been tested for their stimulatory activity with none of the isolated substances singly or in mixtures eliciting as strong a response as the original extract (21). Glycine, the most active of the compounds tested, did possess marked stimulatory activity in solutions of 10^{-3} M. Considering the evidence suggesting that response-inducers are often proteins (22, 23, 24), a series of glycine peptides were assayed for activity. However, as can be seen in Table 5, glycine proved to be at least ten times more active than any of the peptides tested.

An exciting possibility emanating from the work of Carr et al. (23) is the probable presence of a response-inducing low molecular weight polypeptide in shrimp extract. If such a polypeptide were isolated and sequenced it would allow the analysis of chemorecption in Nassarius obsoletus at a molecular level. The present work represents a joint effort

TABLE 5

THE STIMULATORY ACTIVITY OF GLYCINE PEPTIDES[a]

Substance	Molecular Weight	Conc. (M)	No. Test Animals	% Response
Glycine	75	10^{-2}	20	75
		10^{-3}	20	50
		10^{-4}	10	30
N-acetyl glycine	117	10^{-2}	10	0
Ethyl ester glycine HCl	103	10^{-2}	10	0
Glycyl phenylalanine	222	10^{-2}	20	40
		10^{-3}	10	20
Glycyl glycine	132	10^{-2}	20	45
		10^{-3}	10	10
Triglycine	189	10^{-2}	20	0
Tetraglycine[b]	246	10^{-2}	10	0
Pentaglycine[b]	303	10^{-3}	10	0
Hexaglycine[b]	360	10^{-2}	10	0

[a]Unpublished data, work done by Nancy Danoff and Cary Thrall.

[b]Made soluble in sea water by warming and adding Na_2HCO_3 to pH 8.

by William Carr, Samuel Gurin, and Elizabeth Hall to isolate and characterize the major response-inducing molecule(s) from shrimp extract.

CHAPTER VI
SIZING THE MAJOR RESPONSE-INDUCER(S) FROM SHRIMP EXTRACT

There are a variety of techniques available to the biochemist for approximating the molecular weight of specific substances. Several of these techniques were employed in estimating the size of the major stimulatory molecule(s) found in the muscle extracts of the shrimp, Penaeus duorarum, specifically, ammonium sulfate precipitation, ultrafiltration through an Amicon UM 2 membrane, and Sephadex G-25 and G-10 chromatography.

Preparation of Shrimp Extract

Aqueous extracts of the shrimp, Penaeus duorarum, were prepared by gently shaking coarsely minced shrimp muscle with 3 volumes of cold water. After shaking for 30 min in an ice bath, the solution was centrifuged for 30 min at 10,000 rpm in a Beckman J-21 refrigerated centrifuge. The clear supernatant was decanted and tested for activity. The solution was highly stimulatory with only 0.16 µl of solution per ml of sea water necessary to induce the proboscis search reaction in 50% of the test animals (effective dose for 50% of test animals = ED_{50}).

Ammonium Sulfate Precipitation

Eighteen ml of saturated ammonium sulfate (0.7 g of ammonium sulfate per ml of water) were slowly added to 2 ml of prepared shrimp extract and allowed to sit overnight at 7°C. The resulting 90% ammonium sulfate solution was then centrifuged for 30 min at 10,000 rpm at 0°C. The precipitate was washed with saturated ammonium sulfate solution, redissolved in two ml of water, and tested for its activity. As illustrated in Table 6, only 18% of the biological activity was precipitable in this manner suggesting that either the major stimulatory factor(s) in shrimp extracts is non-protein in character or is of low molecular weight. Similar experiments were run yielding comparable results.

Ultrafiltration

Another portion of the prepared shrimp extract (38 ml) was ultrafiltered through an Amicon UM 2 membrane at 4°C and 35 psi of nitrogen to a retenate volume of 4 ml. The retentate and ultrafiltrate were brought up to the original volume of 38 ml and bioassayed. Table 6 shows that the biological activity was rather evenly distributed between the retentate, with 57% of the original activity, and the ultrafiltrate, with 35% of the original activity. This indicated that the major response inducer(s) is within the threshold range of the pore sizes of an Amicon UM 2

TABLE 6

PORTION OF TOTAL ACTIVITY OF SHRIMP EXTRACT
IN AMMONIUM SULFATE PRECIPITATE
AND ULTRAFILTRATION FRACTIONS

Material Assayed	ED_{50} (µl/ml)[a]	% Recovery[b] of Activity
Total shrimp extract (1:3)	0.16	100
Ammonium sulfate ppt.	0.88	18
UM 2 Retentate	0.26	62
UM 2 Ultrafiltrate	0.46	35

[a] ED_{50}: The volume of active solution in 1 ml sea water (or the weight of substance dissolved in 1 ml sea water) required to yield a 50% response of the test animals.

[b] The % of the original activity recovered after any one fractionation procedure was calculated from the ED_{50}'s as follows:

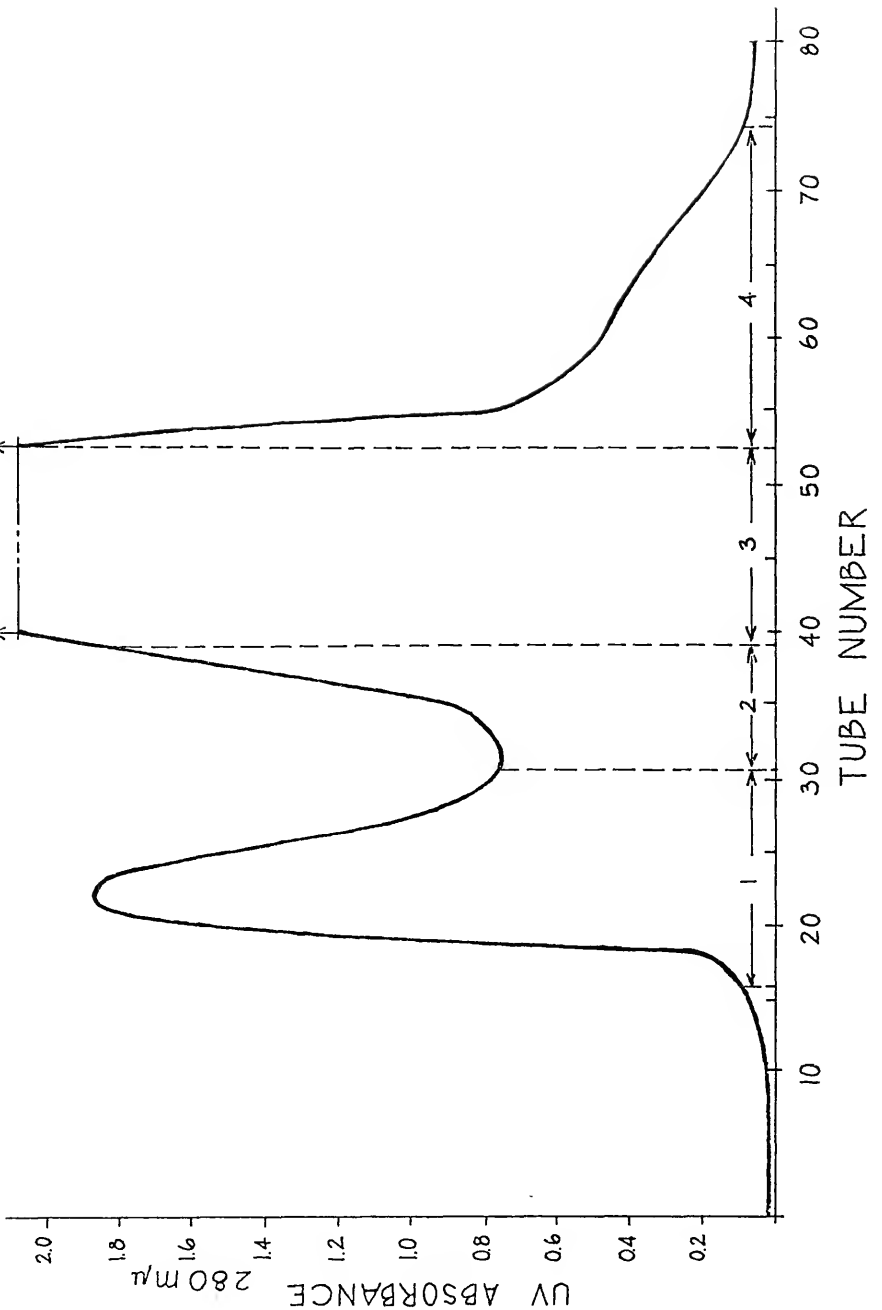
$$\frac{\frac{\text{Total vol (wt) of fractionated sample}}{ED_{50} \text{ of fractionated sample}}}{\frac{\text{Total vol (wt) of original sample}}{ED_{50} \text{ of original sample}}} \times 100\% = \% \text{ Recovery of Activity}$$

membrane. Such substances would have a molecular weight of at least several hundred and probably less than two thousand. Additional ultrafiltration data further indicate that the molecular size of the major response inducer(s) is within the threshold range of the membrane.

Sephadex Chromatography

In an effort to further isolate the stimulatory molecule(s), a more concentrated shrimp extract (prepared from 1.5 volumes of water per volume of shrimp) was chromatographed on a Sephadex G-25 column, which will fractionate molecules ranging from 1,000 to 5,000 in molecular weight. Ten ml of shrimp extract were applied at room temperature to a Sephadex G-25 column (5 x 30 cm) that had been swollen in phosphate buffer and equilibrated with distilled water. The shrimp extract was eluted with distilled water at a flow rate of 30 ml/hr. The eluate was collected in uniform samples (100 drops each) and the UV 280 absorbancd recorded as seen in Figure 12. Individual samples were pooled to make four fractions as indicated in Figure 12. Each fraction was lyophilized, redissolved in 10 ml of distilled water and bioassayed. The concentration of protein in each fraction was estimated by the procedure of Lowry (25). Fraction 1 corresponded to the void volume as determined by chromatographing Blue Dextran (mw = 2×10^6). The included fraction, Fraction 3, contained 89% of the activity present in

Figure 12. Chromatographic separation of shrimp extract on a Sephadex G-25 column. Uniform samples of eluate (100 drops each) were collected in individual tubes and the tube number plotted against the recorded UV 280 absorbance of the eluate. Samples contributing to the UV 280 peaks were pooled as follows: Fraction 1, tubes 16 through 30; Fraction 2, tubes 31 through 38; Fraction 3, tubes 39 through 52; and Fraction 4, tubes 53 through 74.


UV ABSORBANCE 280 mμ
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
60
70
80
TUBE NUMBER
1
2
3
4

the combined Fractions 1 through 4 (see Table 7). However, Figure 12 illustrates the fact that the column was overloaded, thus there was no fractionation of the included substances.

In order to obtain additional information on the molecular size of the stimulant(s), 2 ml of shrimp extract were similarly chromatographed on a Sephadex G-10 column (2.4 x 47 cm). Four ml samples were collected and analyzed for their UV 280 absorbance. The samples contributing to each of the three UV 280 peaks shown in Figure 13 were pooled to make three fractions. Bioassaying the fractions showed 56% of the original activity was recovered in Fraction 3, the included fraction, with very little activity found in Fractions 1 and 2. An amino acid analysis of Fraction 3 showed a significant concentration of amino acids present. These results indicate that the stimulatory molecule(s) is of low molecular weight and cannot be readily separated from the amino acids present by Sephadex chromatography.

TABLE 7

FRACTIONS OF SHRIMP EXTRACT ELUTED
FROM A SEPHADEX G-25 COLUMN

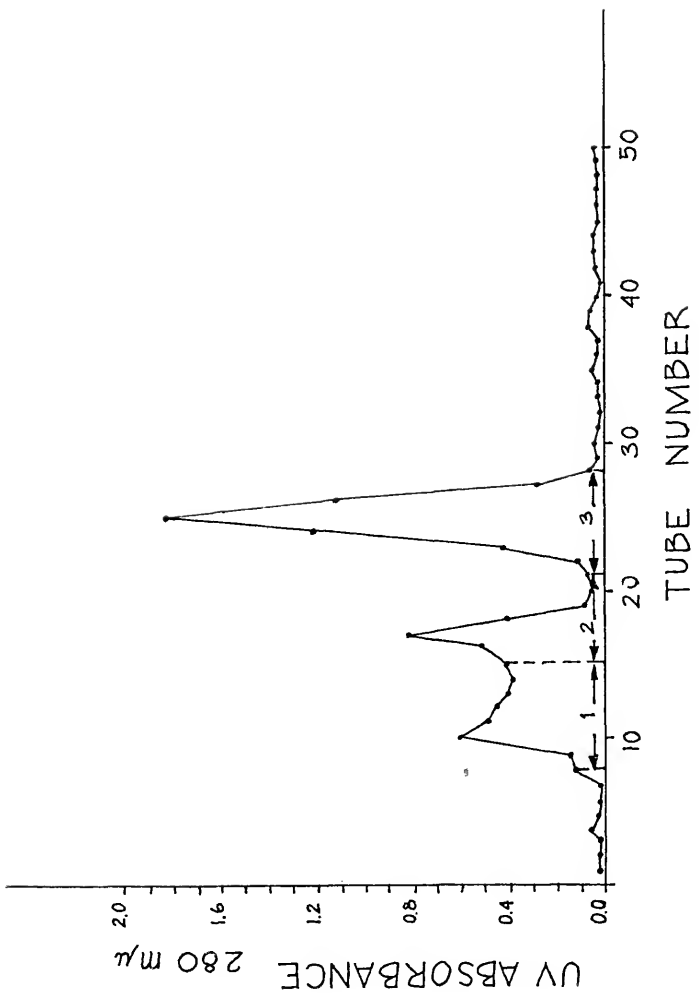
Fraction	Prot. Conc. (mg/ml)	Total Protein (mg)	ED_{50}[a] (µg/ml)	% Recovery[b] of Activity
Original sample	6.72	67.2	1.34	100
Mixture of fractions 1-4	3.3[c]	33.0	0.81	81
Fraction 1	2.85	28.5	5.70	10
Fraction 2	0.085	0.85	----	---
Fraction 3	0.255	2.55	0.07	73
Fraction 4	0.11	1.1	2.75	1

[a] ED_{50}: Defined in Table 6.

[b] % Recovery of Activity: Calculated as described in Table 6.

[c] The protein concentration of the mixture was less than that of the original for some of the lyophilized protein (especially in Fraction 1) did not redissolve.

Figure 13. Fractionation of shrimp extract on a Sephadex G-10 column. The eluate was collected in tubes of 4 ml samples and the tube number plotted against the recorded UV 280 absorbance. The samples contributing to the three UV 280 peaks were pooled as follows: Fraction 1, tubes 8 through 14; Fraction 2, tubes 15 through 20; and Fraction 3, tubes 21 through 28.


UV ABSORBANCE 280 mμ
2.0
1.6
1.2
0.8
0.4
0.0
10
20
30
40
50
TUBE NUMBER
1
2
3

CHAPTER VII
ISOLATION AND CHARACTERIZATION OF THE MAJOR RESPONSE-INDUCER(S) FROM SHRIMP EXTRACT

Preparation of Shrimp Extract (Preparation I)

Several preliminary experiments were performed to determine whether the stimulatory activity of aqueous extracts of shrimp could be precipitated with methanol. Freshly minced shrimp muscle (50 gm) was stirred 1 hr at 3°C in 2% NaCl solution (50 ml). After centrifugation cold methanol was added to the clear supernatant to a final concentration of 75% methanol (v/v). The resulting precipitate was chilled in a deepfreeze overnight, collected by centrifugation, and bioassayed. The precipitate was found to contain no more than 10-15% of the original activity, while the 75% aqueous methanolic extracts usually contained 80-90% of the original activity. The residue, left following evaporation of the 75% methanol, was washed with absolute acetone and then extracted repeatedly with cold absolute methanol. When the methanol had been evaporated and the residue dissolved in water it was found to have 70-75% of the original activity. Several such preparations were combined to yield 20 ml of a clear, slightly yellow solution. This solution was vigorously shaken with 3-4 ml of chloroform for

a few minutes and centrifuged. The aqueous fraction was collected and aerated for several minutes to remove residual chloroform. All color was removed by the chloroform; nevertheless the clear aqueous phase was filtered by gravity through a small wet filter to remove any denatured insoluble protein. The resultant solution which retained full activity (preparation I, ED_{50} = 0.06 µl/ml) was used for all subsequent studies. See Figure 14.

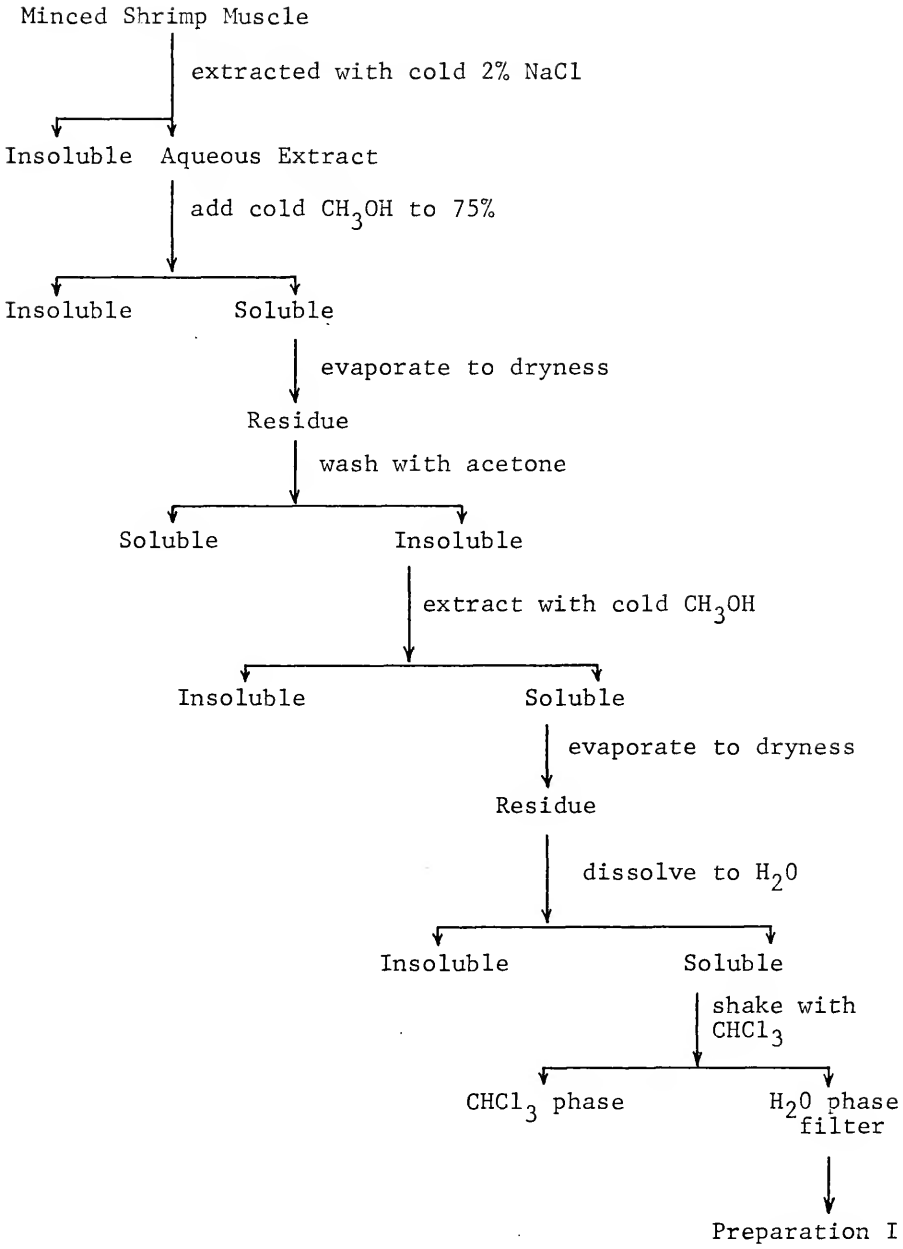
An amino acid analysis of preparation I by the Stein-Moore technique indicated the presence of trace quantities of most amino acids with glycine, arginine, alanine, serine, and proline present in significant amounts.

Enzymatic Digestion Experiments

In order to determine whether the shrimp stimulatory factor(s) has the properties of a small peptide, a series of enzymatic digestion experiments were performed. Aliquots of preparation I (0.6 ml) were diluted to 5 ml and incubated respectively with trypsin, carboxypeptidase, and aminopeptidase at pH 7 for 1 hr at room temperature. All enzymes were highly purified Worthington preparations.

After incubation each solution was shaken vigorously for 2-3 min with 1 ml of chloroform. The suspensions were allowed to settle and the supernatant fluid filtered by gravity through a small wet filter paper. The chloroform phases were washed several times with 1 ml of water and refiltered. The aqueous fractions were collected and

Figure 14. Flow sheet for the fractionation of preparation I.


Minced Shrimp Muscle
extracted with cold 2% NaCl
Insoluble
Aqueous Extract
add cold CH_3OH to 75%
Insoluble
Soluble
evaporate to dryness
Residue
wash with acetone
Soluble
Insoluble
extract with cold CH_3OH
Insoluble
Soluble
evaporate to dryness
Residue
dissolve to H_2O
Insoluble
Soluble
shake with $CHCl_3$
$CHCl_3$ phase
H_2O phase
filter
Preparation I

aerated until the odor of chloroform could no longer be detected. Each fraction was then adjusted to 10 ml and bioassayed. The results seen in Table 8 reveal only a slight decrease in activity after trypsin digestion; however, digestion with aminopeptidase resulted in a 70% decrease in biological activity. This suggests that the active principal is a peptide.

Two-dimensional Chromatography and Electrophoresis

A sample of preparation I as well as preparation I which had been previously digested with aminopeptidase were then concentrated *in vacuo* and spotted on a large sheet of Whatman 1 paper. Two dimensional chromatography and electrophoresis were then employed. Vertical chromatography was performed using a 4:1:5 butanol:acetic acid:water solvent system; subsequent electrophoresis of the sheet was run in pyridine acetic acid buffer (pH 4.8) at 2000 V for 1 hr. Upon staining, preparation I revealed four distinct spots in the anionic region; two spots were identified as glutamic and aspartic acids and the other two were unknown substances. The sample of preparation I previously digested with aminopeptidase lacked one of the unknown spots, with the other unknown diminished in intensity. These results suggest that the active substance(s) is a peptide which is anionic in character. See Figure 15.

TABLE 8

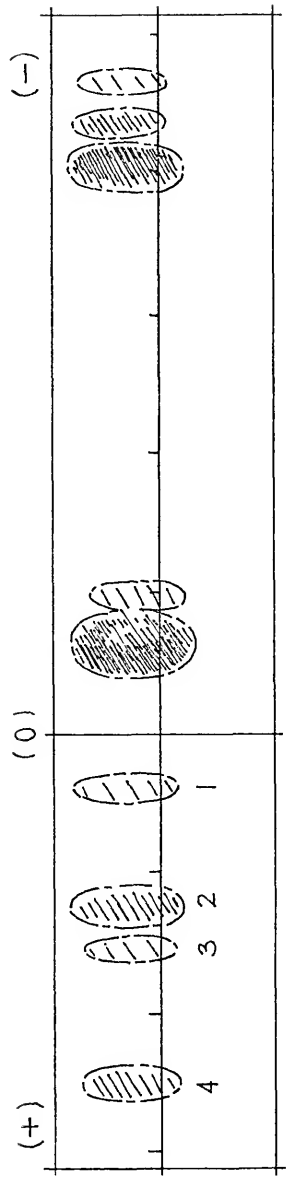
ENZYMATIC DIGESTIONS OF PREPARATION I

Enzyme	ED_{50} (µl/ml)[a]	% Recovery[b] of Activity
Preparation I	0.06	100
Preparation I + Aminopeptidase	0.20	30
Preparation I + Trypsin	0.08	75
Preparation I + Carboxypeptidase	0.10	60

[a] ED_{50}: Defined in Table 6.

[b] % Recovery of Activity: Calculated as described in Table 6.

Figure 15. Ninhydrin positive spots obtained by electrophoresing preparation I. The anionic region (left of the origin) included four ninhydrin positive substances labeled on the illustration as 1, 2, 3, and 4. The substances corresponding to spots 1 and 3 were designated peptide 1 and peptide 2 and the substances corresponding to spots 2 and 4 were identified as glutamic and aspartic acid.


(-)
(0)
(+)
1
2
3
4

Electrophoresis and Elution of Preparation I

To provide additional evidence that the stimulatory substance(s) is indeed anionic, the anionic, cationic, and neutral fractions were eluted and bioassayed. A large sheet of Whatman 1 paper was spotted with six spots of preparation I (0.2 ml each) and subjected to electrophoresis at 2000 V in pyridine acetate buffer at pH 4.7 for 1 hr. The paper was then cut into six strips, each corresponding to one of the spots of preparation I. The top and bottom strips were stained with ninhydrin to show some amino acids and peptides. See Figure 15. Two of the remaining strips were cut vertically to separate the anionic, cationic, and neutral fractions. Each section was separately eluted with 50 ml of millipore-filtered sea water by passing the eluting fluid dropwise through the strip. As seen in Table 9, the anionic sections yielded stimulatory solutions, 50% of the original activity one time and 33% the other time. No activity was recovered from the cationic and neutral fractions.

The final two remaining strips were combined and cut vertically to separate the four anionic substances seen in Figure 15 and designated as follows: peptide 1, glutamic acid, peptide 2, and aspartic acid. The strips containing peptide 1 and peptide 2 were separately eluted with 50 ml of millipore-filtered sea water and assayed. Peptide 2 was quite active, accounting for 65% of the original activity;

TABLE 9

THE RELATIVE ACTIVITIES OF ELUTED FRACTIONS AFTER ELECTROPHORESES OF PREPARATION I

Fraction	ED_{50} (μl/ml)[a]	% Recovery[b] of Activity
Preparation I	0.065	100
Cationic	-----	5
Neutral	-----	5
Anionic 1	0.13	50
Anionic 2	0.20	33
Peptide 3	0.10	65
Peptide 1	0.20	33

[a] ED_{50}: Defined in Table 6.

[b] % Recovery of Activity: Calculated as described in Table 6.

peptide 1 was less active but did account for 33% of the original activity.

CHAPTER VIII
DISCUSSION

It is clear that the substance migrating electrophoretically as peptide 2 contains the bulk of the biological activity originally present in preparation I. It is anionic at pH 4.8 and is readily digested by aminopeptidase. Peptide 1, although less active and more resistant to the action of aminopeptidase, does have significant stimulatory activity. Whether these two peptides are structurally related remains to be established.

It is clear that future experimentation involves the isolation of larger quantities of peptides 1 and 2 either by electrophoresis or by column chromatography in order to establish the following: 1) homogeneity, 2) biological activity on a weight basis, and 3) the amino acid sequence.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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