Experiments in Marine Biochemistry

HOMARINE METABOLISM IN PENAEUS DUORARUM*

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A fractionation procedure has been developed which permits the isolation of 1 to 2 mg of homarine from a single shrimp. This procedure was used to show that homarine is endogenously synthesized by Penaeus duorarum in the free unbound form, and to study the metabolic precursors involved. Injected DL-[14C]tryptophan was not converted to [14C]homarine. However, [6-14C]quinolinic acid, a known catabolite of tryptophan, is an effective precursor. [2-14C]Acetate and [U-14C]glycerol are effectively converted to [14C]homarine while [14C]bicarbonate is poorly utilized. The injection of L-[U-14C]aspartate resulted in labeled homarine, but the quantity converted was less than expected. Since [14C]glycerol is an effective precursor there is a possibility that quinolinic acid may be formed in P. duorarum by a condensation similar to that of glyceraldehyde 3-phosphate with aspartic acid or a closely related metabolite. It is suggested that decarboxylation of quinolinic acid gives rise to picolinic acid which is methylated to yield homarine. L-[methyl-14C]Methionine efficiently provides the N-methyl carbon presumably via S-adenosylmethionine.

Although the natural occurrence of homarine (1-methyl-2-pyridine carboxylic acid) was first reported in 1933 by Hoppe-Seyler (1), the intervening years have provided little information concerning its function, biosynthesis, or metabolic fate. Studies of its distribution in tissues have revealed that it is present in most marine invertebrates below the Echinoderms and absent in terrestrial or freshwater species. Although the concentration of homarine in the shrimp Palaemonetes vulgaris was estimated (2) to range from 0.60 to 1.19 mg/g, wet weight, no trace of homarine was observed in freshwater crayfish (3, 4). Gasteiger et al. (3) have carefully investigated the distribution of homarine in various tissues of Loligo (squid), Homarus (lobster), and Limulus (King crab). In general, highest concentrations were found in nerve, muscle, and glandular tissues (7 to 10 mg/g, wet weight).

Experiments designed to determine a physiological function for homarine have been unsuccessful, i.e. osmotic regulation (5, 6), neurohumoral function (3), muscle contraction (7), pharmacological action on muscle (8), or on the crustacean peripheral nervous system (9).

In 1971, Dall (6) reported that homarine was derived from tryptophan by the shrimp Metapenaeus and that it appeared in shrimp blood linked to a tetrapeptide.

This study was undertaken to determine whether (a) homarine is endogenously synthesized and (b) homarine is present exclusively in its free molecular form or is in part covalently bound. With convincing evidence that homarine is indeed endogenously synthesized, a systematic study was begun to identify the metabolic precursors involved and the biochemical pathways by which homarine synthesis is accomplished.

METHODS

Maintenance of Animals—The selection of the shrimp, Penaeus duorarum, was based on several factors: (a) they are readily accessible, (b) they can be maintained in the laboratory for an indefinite period, (c) they contain workable quantities of homarine, and (d) they are relatively easy to inject. The animals were collected from the Cedar Key area and 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 (Sterneo).

High survival rates were observed when shrimp were injected intravenously (through the articular membrane of the fifth abdominal segment just to the left of the mid-dorsal line) or intramuscularly into the ventral portion of the first abdominal segment.

Chromatographic Methods—At a pH of 10.9, homarine passes unretarded through anion exchange resins while dipolar ions including most amino acids are retained. For this purpose Dowex AG 1-X8 resin (OH− form, 200 to 400 mesh) in a column (2.5 x 21 cm) was equilibrated and eluted with 0.5% NH₄OH (pH 10.9).

Initial experiments demonstrated that homarine is firmly bound to cation exchange resins and can only be eluted after the passage of 15 bed volumes of 0.1 N HCl. A column (1.5 x 28 cm) of Dowex AG 50×-X8 resin (H+ form, 200 to 400 mesh) was poured and washed with a minimum of 5 bed volumes of 2 N HCl. The column was then exhaustively washed until the pH of the effluent was raised to 6. The sample to be chromatographed was applied, the column then washed with 200 to 300 ml of water, and the elution begun with 0.1 N HCl.

Thin layer microcrystalline cellulose plates were also utilized with...
two solvent systems which were found to give satisfactory separation (60/20/20, butanol/acetic acid/water; and 90/5/5, methanol/acetic acid/water). The $R_f$ value of homarine was 0.41 in the acidic butanol system and 0.66 in the acidic methanol system.

Homarine was chromatographed on a Sephadex G-10 column (1 x 33 cm) that was equilibrated and eluted with phosphate buffer (0.01 M, pH 7.5).

Radioactivity Measurements—Isolated homarine preparations dissolved in aqueous solutions ranging from 0.1 to 0.5 ml in volume were counted in a Beckman LS 230 liquid scintillation counter utilizing a toluene-based mixture with 10% v/v of RSO-3 and 0.8%, w/v, of T1A fluor (10). All samples were counted for a minimum of 10 min for five times and the average counts per min calculated. Background counts for 0.1 to 0.5 ml of authentic nonradioactive homarine hydrogen sulfate (1 mg/g) were found to be 31 ± 2 cpm, with a calculated counted efficiency of 91%.

Fractionation and Purification of Homarine—A fractionation procedure calculated to isolate a few milligrams of homogeneous homarine from extracts of shrimp muscle was developed. The following procedure was found to give satisfactory yields and purity (Fig. 1).

Fresh shrimp muscle (5 to 20 g) was blended three times in 100 ml of cold 95% ethanol and centrifuged at 10,000 rpm for 10 min at 3-5°C. The combined supernatant fluid was evaporated to dryness in a rotary evaporator under reduced pressure, backwashed with 5 ml of water, and the two aqueous phases combined.

The resulting residue was dissolved in 5 ml of water, brought to pH 10.9 with dilute NH$_4$OH, and passed through an AG 1-X8 anion exchange column. Previous experiments demonstrated that amino acids such as glycine and tryptophan in addition to the pyridine and quinolinic acids are retained by the column while the n-methyl pyridine carboxylic acids, homarine, and trigonelline pass unretracted.

After elution with approximately 10 bed volumes of 0.5% NH$_4$OH, the eluate was neutralized with dilute HCl, concentrated to approximately 5 ml, and chromatographed on a cation exchange resin, AG 50W-X8 prepared as described previously. Trigonelline was eluted from the column during the water wash, whereas homarine was eluted only after the passage of 700 to 1000 ml of 0.1 N HCl. The eluate was monitored for its ultraviolet absorbance at 274 nm ($\lambda_{max}$ for homarine) and the homarine-containing fraction was subsequently reduced in volume to 1 to 3 ml.

Although the homarine preparation thus obtained appeared to be fairly pure, a final precipitation with phosphotungstic acid yielded a preparation whose properties were identical with those of authentic homarine hydrogen sulfate (ultraviolet absorption and thin layer chromatography in two solvent systems). The concentrated eluate (1 to 3 ml) was chilled, adjusted to approximately 1 N H$_2$SO$_4$ with 6 N H$_2$SO$_4$, and precipitated at 0°C with 10% phosphotungstic acid. After chilling for several hours, the precipitate was recovered by centrifugation, washed with cold 10% phosphotungstic acid containing 1 N H$_2$SO$_4$, and dissolved by dropwise addition of 0.5 N NaOH to pH 5 ± 0.6. The clear solution was acidified again with H$_2$SO$_4$ and reprecipitated with phosphotungstic acid. After centrifugation, the precipitate was redissolved by addition of 0.5 N NaOH; 10% Ba(OH)$_2$ solution was added to precipitate barium phosphotungstate which was removed by centrifugation and the excess Ba$^{2+}$ ions removed by addition of 2 N H$_2$SO$_4$ to a pH of 1 or 2. Following centrifugation or filtration, a colorless solution of pure homarine hydrogen sulfate is obtained (see Fig. 1 for flow diagram).

The absorption spectrum of isolated homarine hydrogen sulfate is identical with that of synthetic recrystallized homarine hydrogen sulfate, for both specimens $\lambda_{max}$ = 274 nm and $\lambda_{min}$ = 240 nm at pH 1. The extinction coefficient reported for synthetic homarine (4) is 6300, and this value was utilized to calculate homarine concentrations in subsequent preparations.

Synthetic and isolated homarine yielded identical $R_f$ values when run in the two separate solvent systems on thin layer plates. Table I demonstrates that in the acidic butanol system homarine separates readily from nicotinic and picolinic acids but not from trigonelline. In the acidic methanol system homarine is well separated from all three. Homarine isolated by the fractionation method described above yields only one ultraviolet absorbing spot in each case, and the $R_f$ values are identical with synthetic homarine in both solvent systems. The ultraviolet absorbing spot also gives a yellow color when sprayed with alkaline a-naphthol (equal volumes of 5 N NaOH and 1% a-naphthol in ethanol) as described by Leonard and MacDonald (4). No ninhydrin-reacting compounds were detected on these chromatograms.

**RESULTS AND DISCUSSION**

**Endogenous or Exogenous Origin**—The question of whether homarine is synthesized by shrimp or derived from their food was investigated by feeding shrimp a diet devoid of homarine. Twelve shrimp were fed frozen crayfish as their sole diet for 21 days. At weekly intervals three shrimp were removed and their homarine content estimated. Average values were calculated for each group. The results obtained were as follows: original

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_f$</th>
</tr>
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<tbody>
<tr>
<td>Homarine</td>
<td>0.41</td>
</tr>
<tr>
<td>Trigonelline</td>
<td>0.42</td>
</tr>
<tr>
<td>Picolinic acid</td>
<td>0.57</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.73</td>
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**Table I**

<table>
<thead>
<tr>
<th>Solvent System I*</th>
<th>Compound</th>
<th>$R_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homarine</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>Trigonelline</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>Picolinic acid</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.73</td>
<td></td>
</tr>
</tbody>
</table>

**Solvent System II**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$R_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homarine</td>
<td>0.66</td>
</tr>
<tr>
<td>Trigonelline</td>
<td>0.54</td>
</tr>
<tr>
<td>Picolinic acid</td>
<td>0.78</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.83</td>
</tr>
</tbody>
</table>

* Solvent System I: butanol/acetic acid/water, 60/20/20.
* Solvent System II: methanol/acetic acid/water, 58/5/5.
controls (0 days), 0.69 ± 0.19 mg/g; 1 week, 0.68 ± 0.28 mg/g; 2 weeks, 0.83 ± 0.22 mg/g; 3 weeks, 0.51 ± 0.15 mg/g. Although considerable variation was observed within each group, the average values suggest little change in total homarine content over the period studied. It was therefore concluded that homarine is endogenously synthesized. This conclusion was confirmed by subsequent radioisotope studies.

Free or Bound Homarine—Dall (6) has suggested that homarine appears to be bound to a small peptide in the shrimp, Metapenaeus, and that the homarine and proteins found in the hemolymph of the same species are inseparable by Sephadex G-10 chromatography. In an effort to clarify the latter observation, samples of synthetic and isolated homarine were separately mixed with blue dextran (MW 2 × 10⁶) and each preparation chromatographed on a Sephadex G-10 column. Both preparations were clearly separated by this technique.

If homarine is wholly or partially bound to protein or polypeptides, it should appear in the 95% ethanol-insoluble precipitate (first step in flow diagram) and be subsequently released by hydrolysis. Following hydrolysis of such material with 6 N HCl at 100° for 28 hours, the solution was suitably concentrated and chromatographed on a cation exchange column (AG 50W-X8). The ultraviolet absorbance at 274 nm within the region of homarine elution represented less than 1% of the region subsequently isolated from the ethanol-soluble fraction. Homarine is therefore not bound to alcohol-insoluble proteins or peptides.

The alcohol-soluble fraction containing the homarine was evaporated to dryness and the residue thoroughly extracted with water. Aliquot portions of the aqueous solution were chromatographed on thin layer plates in two solvent systems and scraped off the plates, extracted with water, and re-assayed for radioactivity. The homarine fraction was lost before it could be chromatographed.

The close chemical relationship of picolinic acid to nicotinic acid and homarine to trigonelline has led to suggestions that homarine is a product of tryptophan catabolism (4). In a preliminary experiment with DL-[14C]tryptophan it was observed that 24 hours after intravenous injection, the animal yielded a crude homarine preparation which was radioactive. This radioactivity was largely lost when the sample was rechromatographed with cold carrier tryptophan. It was apparent that the crude homarine was contaminated with radioactive tryptophan and that extreme care was required to ensure complete removal of impurities. In all subsequent experiments with radioactive precursors, the complete fractionation procedure described in the flow diagram was followed. Whenever radioactive homarine was recovered, it was subjected to thin layer chromatography in two solvents, scraped off the plates, extracted with water, and re-assayed for radioactivity (Table II).

Two separate experiments were run with 10 and 50 μ Ci of DL-[14C]tryptophan uniformly labeled in the benzene moiety of the indole ring. In both experiments the recovered homarine contained no radioactivity (Table II). Both homarine preparations gave the same Rf values in two solvent systems when compared with synthetic homarine and, furthermore, had identical ultraviolet spectra. It appears that tryptophan is not converted by this species to homarine, or, at best, to only a minor extent.

[2-14C]Acetic acid, 125 μCi, was injected into a single animal in two equal portions 12 hours apart. After 24 hours the animal was killed and the homarine isolated. Then 4.5 mg of homarine were recovered containing 2220 dpm with a specific activity of 493 dpm/mg. Aliquots of this homarine preparation were chromatographed on thin layer plates in two solvent systems and retained their radioactivity as seen in Table II. It is clear from this experiment that (a) shrimp do indeed synthesize homarine, and (b) tryptophan is not a significant precursor of homarine since it has been established that [14C]acetate is not a precursor of tryptophan in shrimp (11).

### Table II

Activity of homarine fractions isolated from shrimp injected with labeled precursors

<table>
<thead>
<tr>
<th>Labeled compound*</th>
<th>No. of animals injected*</th>
<th>Amount injected</th>
<th>Total radioactivity recovered</th>
<th>Homarine recovered</th>
<th>Specific activity ²</th>
<th>Specific activity of chromatographed homarine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μCi</td>
<td>dpm</td>
<td>mg</td>
<td>dpm/mg</td>
<td>Fraction I ²</td>
</tr>
<tr>
<td>DL-[benzene ring-U-14C] Tryptophan</td>
<td>3</td>
<td>10</td>
<td>0</td>
<td>7.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>50</td>
<td>0</td>
<td>2.2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>[2-14C] Acetic acid</td>
<td>1</td>
<td>125</td>
<td>2220</td>
<td>4.5</td>
<td>490</td>
<td>420</td>
</tr>
<tr>
<td>[6-14C] Quinolinic acid</td>
<td>1</td>
<td>15</td>
<td>880</td>
<td>0.8</td>
<td>1130</td>
<td>1410</td>
</tr>
<tr>
<td>[1-U-14C] Aspartic acid</td>
<td>3</td>
<td>25</td>
<td>121</td>
<td>16.9</td>
<td>7.1</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>100</td>
<td>70</td>
<td>1.9</td>
<td>40</td>
<td>--</td>
</tr>
<tr>
<td>[U-14C] Glycerol</td>
<td>2</td>
<td>50</td>
<td>1930</td>
<td>2.9</td>
<td>630</td>
<td>675</td>
</tr>
<tr>
<td>[14C] Bicarbonate, sodium salt</td>
<td>2</td>
<td>400</td>
<td>57</td>
<td>2.4</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>1-[methyl-14C] Methionine</td>
<td>2</td>
<td>100</td>
<td>560</td>
<td>3.2</td>
<td>174</td>
<td>151</td>
</tr>
</tbody>
</table>

* Radioactive compounds were purchased from Amersham/Searle and New England Nuclear.

1 The elapsed time between injections and killing of the animals varied from 9 to 24 hours.

² The estimated specific activity of the isolated homarine fractions was calculated using the ultraviolet absorbance at 274 nm of the homarine and its extinction coefficient 6200.

³ The estimated specific activity of the homarine fraction after being chromatographed on cellulose plates in a butanol/acetate acid/water (60/20/20) solvent system. The homarine band was scraped from the plate and extracted with water.

₁ The radioactivity (Table II).

There is the possibility that homarine exists primarily in its free molecular form.
Although tryptophan appears not to be a precursor of homarine, the possibility still remains that quinolinic acid might lose carbon 7 by decarboxylation to yield picolinic acid, which when methylated forms homarine (Fig. 2). Accordingly 15 μCi of [6-14C]quinolinic acid were injected and the animal killed 12 hours later. The isolated homarine had an estimated specific activity of 1127 dpm/mg. Subsequent chromatography in the acidic butanol and acidic methanol solvents did not decrease the activity of the recovered homarine (Table II). The fact that carbon 6 of quinolinic acid is incorporated into homarine appears to support the proposed pathway shown in Fig. 2. Although it is not rigorously established whether quinolinic acid is first methylated and subsequently decarboxylated to form homarine, or whether decarboxylation occurs prior to methylation, we have obtained preliminary evidence indicating that [14C]acetate is converted by shrimp tissue to radioactive picolinic acid. This suggests that the pathway described in Fig. 2 is reasonable, and it is anticipated that this mechanism will be explored more fully.

It is of additional interest that the relatively high specific activity of the isolated homarine preparation suggests that quinolinic acid is an important precursor of homarine. These results suggest also that quinolinic acid is synthesized from a metabolic source other than tryptophan.

It has been proposed by Leete (12) and others (13, 14) that higher plants and certain microorganisms are capable of synthesizing quinolinic acid by a mechanism involving a condensation of glyceraldehyde 3-phosphate with aspartic acid. This pathway was explored by injecting 25 μCi of [1,14C]aspartic acid divided among three shrimp which were sacrificed 9 hours later. The resulting activity in the recovered homarine was lower than expected. A repeat experiment with 100 μCi of labeled aspartic acid administered to a single animal yielded homarine with a specific activity of 40 dpm/mg. Apparently [14C]aspartate can contribute carbon atoms to homarine, yet not as readily as acetate or quinolinic acid. Whether this result can be explained on the basis of dilution by large body stores of aspartic acid or rapid diversion into more metabolic pathways remains to be established. It is more likely that another nitrogen-containing precursor is involved.

[U-14C]Glycerol proved to be considerably more effective. Upon injection of 90 μCi of this substance, 2.9 mg of the subsequently recovered homarine had a specific activity of 630 to 675 dpm/mg. It is apparent that glycerol or a 3 carbon metabolite derived from it can serve as an effective precursor of homarine.

The significant incorporation of acetate carbons into homarine has not been explained. In order to eliminate the possibility that acetate carbons, after ready oxidation to carbon dioxide, are incorporated into homarine via a CO2 fixation reaction, 400 μCi of sodium 14CO2 bicarbonate were injected into two shrimp. Relatively little radioactivity was recovered in the resulting homarine. If it is felt that this result eliminates the possibility that CO2 fixation plays any significant role in the biosynthesis of homarine. This negative result also rules out a theory proposed by Haake and Mantecon (15) who suggested that homarine is formed by carboxylation of N-methyl pyridinium ion. They proposed that homarine could serve as a storage system for CO2, transporting it to extracellular spaces where decarboxylation could occur followed by active transport of the remaining N-methyl pyridinium ion back into the cell.

That the N-methyl carbon of homarine is contributed by methionine is clearly indicated by the results obtained following injection of 50 μCi of L-[methyl-14C]methionine into each of two shrimp. After 12 hours the shrimp were homogenized. The 3.2 mg of recovered homarine had a specific activity of 140 to 175 dpm/mg. The fact that the 14C from the methyl group of methionine was incorporated into homarine provides strong support for the suggestion that S-adenosylmethionine contributes the N-methyl carbon of homarine. Further experimentation will be required to establish (a) the metabolic origin of quinolinic acid in this species, (b) the role of acetate in this biosynthesis, (c) whether other nitrogen containing compounds can more effectively replace aspartic acid as precursors, and (d) the catabolic and metabolic fate of homarine in marine crustacea.

REFERENCES
12. Leete, E. (1965) Science 147, 1000-1006
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