Biosynthesis in Vitro of Homarine and Pyridine Carboxylic Acids in Marine Shrimp

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Mincing and homogenates of muscle obtained from the marine shrimp Penaeus duorarum are capable of synthesizing homarine from [14C]glycine. Glycine carbon atoms are incorporated into homarine but not significantly into picolinate or quinoline. [2-14C]Acetate is readily incorporated into quinolinate in the in vitro system but only slightly into homarine and not at all into picolinate. Quinolinic acid is rapidly methylated to N-methyl quinolinic acid which is not decarboxylated to form homarine. Procedures have been developed for the satisfactory separation of N-methyl quinolinic acid from homarine.

We have previously reported (1) that homarine (N-methyl picolinic acid) is endogenously synthesized by the salt water shrimp Penaeus duorarum. After injection of a number of [14C]-labeled substances and subsequent isolation of radioactive homarine, interpretation of the results was found to be sufficiently difficult to warrant efforts to develop an in vitro system capable of incorporating [14C]-labeled precursors into homarine.

Homogenized shrimp tail muscle was found to be capable of converting [14C]glycine into homarine, while labeled acetate was incorporated into quinolinic acid and not into homarine. When these [14C]-labeled precursors were incubated in the presence of nonlabeled potential intermediates, it was found that two separate biosynthetic pathways were evident. 1) Picolinic and quinolinic acids are not intermediates in the conversion of glycine to homarine; 2) [2-14C]acetate, on the contrary, is incorporated into quinolinic acid and not into homarine. The resulting quinolinic acid is readily methylated to form N-methyl quinolinic acid.

MATERIALS AND METHODS

In Vitro System for Homarine Biosynthesis—The shrimp, P. duorarum, were collected and maintained as previously described (1). After chilling on ice, the inert shrimp were shelled, and the muscle portion was finely minced and blended for 3 to 5 s in ice-cold citrate/phosphate buffer (pH 7.4, 0.06 Molar). Generally 20 ml of the buffer was used with 10 g of shrimp muscle. In typical experiments 10−7 mol each of ATP, DPN, FAD, and MgCl2 were added. Prior to incubation, [14C]-labeled precursors were added, and, on occasion, nonradioactive potential intermediates (usually 5 mg of picolinic acid, quinolinic acid, or N-methyl quinolinic acid) which were subsequently isolated in addition to the homarine. At the end of the incubation (usually 6 h at 20 to 25°C), 5 mg more of nonradioactive carrier was added just prior to the work-up.

Fractionation and Purification—The isolation of purified homarine has been previously described (1). Cell homogenates were deproteinized either 1) by addition of 10 or more volumes of methanol followed by overnight chilling, centrifugation, and evaporation of the aqueous methanolic supernatant fluid to a small volume or 2) by precipitation with 20% trichloroacetic acid. Following the methanol procedure, repeated vigorous shaking with a small volume of chloroform (0.1 to 0.2 volume) yielded a protein-free solution. All subsequent fractionations described in this paper were performed on this solution.

Separation of Homarine and N-Methyl Quinolinic Acid—Following deproteinization by the methanol method (and shaking with chloroform) the concentrated aqueous preparation was evaporated in vacuo to 2 to 3 ml, adjusted with a few drops of concentrated NH4OH to pH 10.5, and chromatographed on a column (1.5 × 20 cm) of BioRad AG 1-X8 resin (OH− form). 100 to 300 mg were subsequently eluted with 0.5% NH4OH. The column was eluted with the same solvent until homarine, N-methyl quinolinic acid, and other UV-absorbing quaternary nitrogen compounds were removed. After evaporation of NH3, the resulting syrup was adjusted to pH 4 to 5 with dilute HCl and evaporated to a volume of 1 to 2 ml. The solution was then chromatographed on a column (1.5 × 20 cm) of BioRad AG 50W-X8 resin (H+ form); the column was thoroughly washed with water and eluted with 0.01 n HCl (400 to 600 ml required). Both homarine and NMQ1 are simultaneously eluted. Following elution of the eluate to 1 to 3 ml, both substances are co-precipitated by phosphotungstic acid in 1 × H2SO4. The mixture, after removal of phosphotungstate, cannot be separated by thin layer chromatography with the solvent systems previously employed (1). Separation of the two substances can, however, be achieved by column chromatography on SP-Sephadex C-25 resin in 0.01 n HCl at pH 2. Homarine is readily eluted with 1 to 2 bed volumes of 0.01 n HCl, while NMQ can only be removed with 1 to 2 bed volumes of 0.1 M NaCl in 0.01 n HCl. Satisfactory separation of the two substances can also be obtained by thin layer chromatography (Analtech MN 300 micocrystalline cellulose) with isopropanol/water (85/15); Rf for homarine is 0.41 and for NMQ is 0.25. It should be emphasized that trace contamination of the recovered homarine and NMQ by radioactive precursors was prevented by repeated additions of nonradioactive carrier precursors at suitable stages in the isolation procedure (prior to AG-50 column chromatography, phosphotungstic acid precipitation, and prior to final chromatography on SP-Sephadex columns). Highly radioactive products were checked several times by such washing-out procedures and rechromatographed for final 14C-labeled measurements.

Picolinic Acid—After incubation of [14C]glycine or [14C]acetate in the presence of carrier picolinic acid and deproteinization with trichloroacetic acid, the picolinic acid was precipitated along with homarine by phosphotungstic acid in 1 × H2SO4, and chilled several hours; after centrifugation, the precipitate was dissolved in dilute NaOH to pH 7 and decomposed with 10% Ba(OH)2 solution. Barium ions were removed with dilute H2SO4, and after evaporation of the aqueous fraction, the residue was extracted into a small volume of methanol to eliminate salts. The methanol solution was evaporated to dryness, and the residue was dissolved in 1 to 2 ml of 0.5% NH4OH (pH 10.9) and chromatographed on a column of BioRad AG 1-X8 resin as previously described. The column was eluted with 0.5% NH4OH until homarine and other quaternary nitrogen compounds were completely removed. After washing with H2O, picolinic acid was eluted with 1 to 4 bed volumes of 0.05 n HCl. Fractions were monitored by UV absorption.

1 The abbreviation used is: NMQ, N-methyl quinolinic acid.
absorbance at 264 nm (\(\lambda_{max} = 264 \text{ nm} ; \lambda_{min} = 244 \text{ nm} ; \epsilon = 6450 \text{ m}^{-1} \text{ cm}^{-1}\) for the HCl salt in H2O).

The picolinic acid fraction was evaporated to dryness and dissolved in minimal HCl; the pH was adjusted to 2 to 3, and the solution was chromatographed on a column (1.5 × 20 cm) of Bio-Rad AG 50W-X8 resin (H+ form, 100 to 200 mesh). After subsequent washing with 250 ml of 0.1 N HCl, picolinic acid was eluted with 3 to 5 bed volumes of 1 N HCl. Following evaporation of the eluate, the residue was thoroughly dried and converted to the methyl ester by refluxing in methanolic HCl for 5 to 6 h. Solvent was removed by evaporation in vacuo, free base was liberated with NaHCO3, and methyl picolinate was extracted into benzene. After drying over MgSO4, the benzene was evaporated, and the residue was dissolved in 0.5 ml of methanol for chromatography on a Hewlett-Packard model 400 gas-liquid chromatograph equipped with a flame ionization detector (6-foot column in minimal H2O; the pH was adjusted to 2 to 3, and the solution was chromatographed on a column (1.5 × 20 cm) of Bio-Rad AG 50W-X8 anion exchange resin in 0.5% NH4OH effectively removed homarine and other quaternary bases; the column was then successively washed with 200 ml of H2O, 500 ml of 0.03 m formic acid, 250 ml of 0.1 m formic acid, and finally with 650 ml of 1.0 m formic acid. Quinolinic acid was eluted with 5 to 9 bed volumes of 1.0 m formic acid, and the fractions were assayed by UV absorbance (\(\lambda_{max} = 266 \text{ to } 268 \text{ nm} ; \lambda_{min} = 258 \text{ to } 260 \text{ nm} ; \epsilon = 5300 \text{ m}^{-1} \text{ cm}^{-1}\) in 1.0 m formic acid).

After removal of the formic acid by evaporation in vacuo at 35°C, the residue was dissolved in 2 to 3 ml of dilute H2SO4 at pH 3 and converted to the Cu(II) salt by addition of powdered CuSO4 with stirring; a precipitate of crystalline copper quinolate formed within a few minutes. After centrifugation, the precipitate was washed once with a small volume of dilute H2SO4 (pH 3) and decomposed with dilute NaOH. The resulting slightly soluble Cu(OH)2 was removed by centrifugation, the aqueous phase was acidified to pH 3 with dilute H2SO4 and evaporated to a dry syrup, and Na2SO4 was precipitated with methanol. The methanol-soluble fraction was evaporated, dried at 1 to 2 mm Hg, and converted to the dimethyl ester as described for picolinic acid. The dimethyl quinolinate was subjected to gas-liquid chromatography as above (2) and collected by means of an effluent splitter and trapped at -77°C, then dissolved in methanol for quantitative UV assay at 264 nm (\(\epsilon = 3200 \text{ m}^{-1} \text{ cm}^{-1}\) in CH3OH). An aliquot was evaporated to dryness for radioassay in a Beckman LS 230 liquid scintillation counter.

**Quinolinic Acid**—Since quinolinic acid is not precipitated by phosphotungstic acid, this step was omitted. Chromatography on AG 1-X8 anion exchange resin in 0.5% NH4OH effectively removed homarine and other quaternary bases; the column was then successively washed with 200 ml of H2O, 500 ml of 0.03 m formic acid, 250 ml of 0.1 m formic acid, and finally with 650 ml of 1.0 m formic acid. Quinolinic acid was eluted with 5 to 9 bed volumes of 1.0 m formic acid, and the fractions were assayed by UV absorbance (\(\lambda_{max} = 266 \text{ to } 268 \text{ nm} ; \lambda_{min} = 258 \text{ to } 260 \text{ nm} ; \epsilon = 5300 \text{ m}^{-1} \text{ cm}^{-1}\) in 1.0 m formic acid).

**RESULTS**

**Pyridine Carboxylic Acids**

**TABLE I**

| Precursor | Homarine Carrier picolin- inate Carrier quinolinate |
|-----------|-----------------------------------------------|
| 3-14C]-Glycine | 346 | 200 |
| 1-14C]-Glycine | 389 |
| 1-14C]-Glutamate | 40 | 132 |
| 1-14C]-Aspartate | 35 |
| 3-14C]-Ala- line | 50 |
| 1-14C]-Serine | 0 |
| 1-14C]-Formate | 78 |
| 1-14C]-Sarcosine | 149 |
| 1-14C]-Sarcosine' | 46 |
| 1-14C]-Lysine | 0 |
| 1-14C]-4-Amino-n-butyr ic acid | 52 |

* 10 to 25 pCi were used.
* Samples were counted in a scintillation counter for a minimum of five 10-min intervals; efficiency was 70%.
* Average of nine experiments.
* Two experiments.
* Diluted with 45 mg of carrier sarcosine.

**TABLE II**

| Precursor | Quinoline | NMQ | Homarine |
|-----------|-----------|-----|----------|
| 3-14C]-Acetate | 150 | 602 | 20 |
| 6-14C]-Quinolate | 9290 | 60 |
| DL-7a-[14C]-Tryptophan | 158 |
| 1-14C]-Glyceraldehyde | 757 |
| 1-14C]-Aspartate + glycerol | 332 |

* 10 to 25 pCi were used.
* Samples were counted in a scintillation counter for a minimum of five 10-min intervals; efficiency was 70%.
* Average of five experiments.

Glycine carbons are incorporated into homarine without intermediate formation of either picolinate or quinolinate. In a preliminary experiment we have observed that dipicolinic acid (2,6-pyridine dicarboxylic acid) is likewise not an intermediate.

**In vitro biosynthesis of homarine**

In an earlier report (1) labeled acetate injected into live shrimp appeared to be incorporated into homarine; when incubated in vitro, however, little or no [2-14C]acetate was converted to homarine (Table II). Significant activity was recovered in carrier quinolinate (none in picolinate). In our earlier in vivo experiments (1) labeled quinolinate also appeared to be a good precursor of homarine. Again, on incubation of [6-14C]quinolate with shrimp homogenate, relatively little radioactivity was recovered in the isolated homarine (Table II). These results suggested that our earlier preparations of radioactive homarine derived from acetate or
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quinolinate were contaminated with traces of a highly radioactive substance. On the theory that quinolinic acid might undergo N-methylation to form N-methyl quinolinate during in vitro incubations, NMQ was chemically synthesized by methylation of quinolinate with methyl iodide. The resulting compound was found to be very similar in its properties to homarine and the two substances separable with difficulty. Radioactive preparations of homarine derived from acetate or quinolinate lost most of their radioactivity upon additional chromatography and elution on SP-Sephadex in 0.01 N HCl, while the NMQ could be subsequently eluted only with 0.1 M NaCl in 0.01 N HCl. As a result of this additional final purification step, the data listed in Table II help to clarify the phenomena under study. 1) Acetate is converted to quinolinate which is readily methylated to NMQ. 2) Labeled tryptophan is converted to quinolinate which in turn methylated. 3) Labeled glycerol, glutamate, and probably aspartate are incorporated into quinolinate. None of the substances listed in Table II appear to be converted to any significant extent into homarine.

DISCUSSION

It is apparent from this work that two separate pathways have been established. 1) Acetate carbon atoms are incorporated into quinolinic acid which is subsequently methylated to NMQ. 2) Conversion of glycine to homarine occurs by a pathway which does not involve picolinate or quinolinate as intermediates. Whether endogenous synthesis of quinolinate (from acetate) by shrimp provides a significant source of nicotinic acid has not been established; it is possible that low or moderate levels of nicotinate may be related to rapid methylation of quinolinate to form NMQ which is then decarboxylated to trigonelline.

Since picolinate is not an intermediate in the biosynthesis of homarine, it may be postulated that glycine condenses with a suitable 4-carbon compound to yield a di- or tetrahydroxypridine carboxylic acid which is subsequently converted to homarine. We have unsuccessfully investigated the possible condensation of glycine with succinic monoaldehyde, hoping that Schiff base formation followed by an aldol type cyclization would yield an important precursor of homarine. Efforts will be continued to find more effective intermediates in this metabolic pathway.

It is recognized that quinolinate is formed by several pathways: 1) from tryptophan in mammals, yeast, and Neurospora (4); 2) from aspartate, acetate, and formate in Clostridium butylicum (5); and 3) from aspartate and glycerol (or a closely related intermediate) in higher plants (6) and various bacteria (7, 8). It would appear that the latter mechanism is similar to what we have observed in shrimp.

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