Mechanism of biosynthesis of trimethylamine oxide in tilapia reared under seawater conditions

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ABSTRACT: The mechanism of biosynthesis of trimethylamine oxide (TMAO) from dietary precursors in a seawater-adapted teleost, Nile tilapia Oreochromis niloticus, was investigated. Diets supplemented with quaternary ammoniums of choline, glycine betaine, carnitine or phosphatidylcholine were administered and significant increases in TMAO levels in the muscle were observed with choline alone. [Methyl-¹⁴C] and [1,2-¹⁴C]-cholines were given through the diet and intraperitoneal injections but [¹⁴C]-TMAO was detected only in fish with dietary administration of [methyl-¹⁴C]-choline. Dietary treatment with [¹⁵N]-choline resulted in the formation of [¹⁵N]-TMAO in the muscle. The incorporation of radioactivity into TMAO was also observed after both dietary administration and intraperitoneal injection of [¹⁴C]-trimethylamine (TMA). There were marked increases in TMA levels when choline was introduced into the isolated intestine. These increases, however, were significantly suppressed in the presence of penicillin. [¹⁴C]-Trimethylamine derived from [methyl-¹⁴C]-choline was detected in the cavity of the isolated intestine. Introduction of [¹⁵N]-choline into the intestinal cavity resulted in the formation of [¹⁵N]-TMA. Reduction activity of TMAO to TMA was observed in intestinal microorganisms under microaerobic conditions. Trimethylamine monoxygenase activity was detected in the liver and kidney. It was concluded that marine teleosts possess the ability to produce TMAO from choline, which is related to intestinal microorganisms and tissue monooxygenase. Furthermore, TMA was suggested to be formed from dietary TMAO by the microbes in the intestine and thus reoxygenated to TMAO by the fish tissue monooxygenase before transfer to the muscle.

KEY WORDS: choline, intestinal microorganism, marine fish, quaternary ammonium, tilapia, trimethylamine monooxygenase, trimethylamine oxide, trimethylamine.

INTRODUCTION

Trimethylamine oxide (TMAO) is a characteristic biological molecule in marine animals. Cod Gadus morhua, sharks, rays, skate and squid possess TMAO at particularly high levels. It is remarkable that appreciable amounts of TMAO were found in the ancestral seawater fish, the ‘living fossil’ coelacanth Latimeria chalumnae (109.4 mmol/L blood and 290 mmol/kg muscle water).¹ However, negligible quantities have been reported in the tissue of freshwater fish.² Trimethylamine oxide has been reported to play a role in cell volume regulation in elasmobranchs, offsetting denaturation of functional proteins induced by urea in sharks;³ it lowers the freezing point of blood in the Antarctic teleosteos Dissostichus mawsoni and Gymnodraco acuticeps;⁴ and it has antioxidant properties.⁵–⁷ Several interesting phenomena related to TMAO in seafood materials have been reported (i.e. ‘green meat’, ‘spongy meat’, trimethylamine [TMA] odor). tuna Thunnus alalunga meat shows a tannish-green color on cooking (‘green meat’ or ‘dead color’), which is disadvantageous in the food industry. Koizumi showed that the greening is closely related to TMAO, SH-groups and myoglobin in cooked tuna meat.⁸ Tuna meat produces green pigment when TMAO levels exceed 8–13 mg/100 g body weight. Formaldehyde formed from TMAO during postmortem storage causes frozen fish fillet to have a spongy texture. The ‘spongy
meat’ decreases textural quality. Formdehyde is formed by the endogenous enzyme TMAO demethylase (EC.4.1.2.32). This enzyme was purified from walleye pollack Theragra chalcogramma muscle and has been characterized in detail. Trimethylamine is a characteristic volatile compound of marine food products. Trimethylamine oxide is readily reduced to TMA by microbial action during postmortem storage. Trimethylamine has a very low odor threshold, for example, the odor thresholds of ammonia, dimethylamine and TMA are 110 000, 30 000 and 600 p.p.b., respectively. Endogenous TMAO in marine fish is not clearly understood, with the exception of TMA as the sole precursor reported in several species. Quaternary ammonium compounds may be TMAO precursors as both have the same moiety (i.e. an N-trimethyl moiety). The effects of intraperitoneal administration of quaternary ammonium compounds have been tested in some marine fish and invertebrates. However, dietary administration of these compounds has not been tested yet. Choline and carnitine are essential substrates in animals and betaines are expected to exist in some marine organisms. Tilapia is a representative euryhaline teleost and is thus found not only in freshwater but also in seawater. It is therefore considered to be a useful model fish species to study TMAO synthesis in seawater teleosts and the relationships between TMAO synthesis and environmental osmolarity. This study was carried out to examine the biosynthesis of TMAO from quaternary ammonium in marine teleosts using Nile tilapia Oreochromis niloticus reared under seawater conditions.

**MATERIALS AND METHODS**

**Animals**

Cultured tilapia were obtained from Kinki University and Ibusuki Fish Research Center of Kagoshima Prefecture and reared in our laboratory. They were fed a commercial diet (Shikoku-Kumiai, Japan). Two-month-old immature fish (body length, 5–6 cm) were used for in vivo tests and 6-month-old fish (body length, 10–15 cm) were used for tests using the isolated intestine and for enzymatic assays. The tilapia were reared in a laboratory aquarium (tank size, 30 cm x 25 cm x 20 cm) containing 25 L of aerated water with a water recirculation system at 27°C. Ground water from our university was used to rear tilapia unless otherwise stated. The anesthetic 0.1% ethylene glycol monophenyl ether was used to kill the fish.

To prepare seawater conditions, an artificial seawater mix (Marine merit, Matsuda, Japan) was added to the aquarium. Seawater-adapted fish were established by adapting the freshwater-reared fish to seawater in a stepwise manner: the salt concentration of the water was increased by one-tenth each day for 10 days. To examine the distribution of TMA and TMAO in the tissues, fish were dissected on the 10th day after completion of seawater adaptation. They were fed a commercial diet during the rearing period and until the day before they were killed and the tissues excised. Each tissue with the exception of the muscle, was pooled, homogenized and subjected to TMAO analysis. The muscles were homogenized individually.

**Chemicals**

Ethylene glycol monophenyl ether was obtained from Wako Pure Chemical Industries (Osaka, Japan). Vitamin-free casein was obtained from Sigma Chemical Co. (Tokyo, Japan). Soluble starch was a kind gift from Dr Masahito Yokoyama (National Research Institute of Fisheries Science, Kanagawa, Japan). Other reagents were obtained from Nacalai Tesque (Kyoto, Japan), and were used without further purification.

**Labeled chemicals**

[Methyl-14C]-choline chloride (specific activity, 54.0 mCi/mmol, 3.7 μmol/mL ethanol) was purchased from Dupont NEN. [Methyl-14C]-choline chloride (55.0 mCi/mmol, 1.8 μmol/mL ethanol), [1,2-14C]-choline chloride (50.0 mCi/mmol, 2.0 μmol/mL ethanol) and [14C]-TMA (5.5 mCi/mmol, 1.8 μmol/mL ethanol) were obtained from American Radiolabeled Chemicals (ARC). [15N]-choline chloride and [15N]-TMA hydrochloride were purchased from Syoko-Tusho (Tokyo, Japan). Choline chloride was dried in an oven at 60°C before injection into the intestine.

**Chemical synthesis of [14C]-TMAO from [14C]-TMA**

Oxygenation of TMA to TMAO and preparation of substrate [14C]-TMAO for injection into isolated intestine were carried out according to the method of Dunstan and Goulding, and Baker and Chaykin, respectively.
Identification of $^{14}$C-TMAO

Thin layer chromatography (TLC) for identification of $^{14}$C-TMAO in the \textit{in vivo} tracer experiments was carried out using cellulose plates.\textsuperscript{20,21}

Identification of $^{14}$C-TMA

A high performance liquid chromatography (HPLC)-flow scintillation analyzer system was used to determine radioactive TMA formed in the perchloric acid extract of the intestine, which was incubated with $^{14}$C-TMAO. It was composed of a Radiomatic Flow Scintillation Analyzer 150TR (Packard Japan, Tokyo, Japan), JASCO 880-PU HPLC (JASCO, Tokyo, Japan) with a 100 μL column loop, a VP-600 integrator (Epson, Japan) and a Shim-pack IC-C3 cation exchange column (4.60 mm ID × 100 mm L) with a Shim-pack IC-GC 3II guard column (Shimadzu, Kyoto, Japan).

Flow liquid scintillation cocktail (ULTIMA-FLO M, Packard Japan) was used for the flow scintillation counter (flow rate, 1.0 mL/min). The mobile phase was 5.0 mM oxalic acid (flow rate, 1.0 mL/min).

The tissue extracts were diluted with distilled water 10 times. The diluted solutions (100 μL) were allowed to diffuse into 500 μL of 0.1 N HCl by adding 1 mL of 6 M KOH using a Conway dish. The HCl solution on an aluminum plate was dried under an infrared lamp (250 W) and dissolved in 200 μL of distilled water. The 200 μL sample was used for TMA analysis with the HPLC system.

Liquid scintillation counting

Radioactivity was measured by liquid scintillation counting using a liquid scintillation cocktail (ATOMLIGHT, Packard Japan) and a WALLAC 1219 liquid scintillation counter (LKB, RACKBETA).

Test diets

Various diets were prepared as shown in Table 1. To the basic mixture was added 29 mmol choline chloride (Chol), glycine betaine hydrochloride (GB), carnitine chloride (Car), trimethylamine hydrochloride (TMA) and trimethylamine oxide dihydrate (TMAO) or 16.5 g phosphatidylcholine (PC). The final weights of the diets were adjusted to 100 g by adding cellulose powder. The final diet mixtures were blended with 110 mL of water, dried at 55°C for 14 h, ground to powder and kept in a freezer at –20°C. The diet without \textit{N}-trimethyl compound was prepared as a control (Basal). To prepare special diets for the tracer experiment, $^{14}$C-labeled choline and $^{14}$C-TMA were added separately to the corresponding diets (Chol and

### Table 1 Compositions of the experimental diets

<table>
<thead>
<tr>
<th>Ingredient (g)</th>
<th>Basal</th>
<th>Chol</th>
<th>GB</th>
<th>Car</th>
<th>PC</th>
<th>TMA</th>
<th>TMAO</th>
</tr>
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<tbody>
<tr>
<td>Vitamin-free casein</td>
<td>41.0</td>
<td>41.0</td>
<td>41.0</td>
<td>41.0</td>
<td>41.0</td>
<td>41.0</td>
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<tr>
<td>α-Starch</td>
<td>15.0</td>
<td>15.0</td>
<td>15.0</td>
<td>15.0</td>
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<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
<td>4.00</td>
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</tr>
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<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
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<tr>
<td>α-Tocopherol</td>
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<td>0.11</td>
<td>0.11</td>
<td>0.11</td>
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<td>4.39</td>
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<td>L-Carnitine chloride</td>
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<td>–</td>
<td>–</td>
<td>5.65</td>
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<td>Phosphatidylcholine</td>
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<td>–</td>
<td>–</td>
<td>16.7</td>
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<td>Trimethylamine hydrochloride</td>
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<td>–</td>
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<td>Trimethylamine oxide dihydrate</td>
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<tr>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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</tr>
</tbody>
</table>

* Amino acid mixture (1.84 g L-arginine hydrochloride, 0.055 g L-histidine, 0.20 g L-isoleucine, 0.35 g L-leucine, 1.25 g L-lysine hydrochloride, 0.55 g L-methionine, 0.30 g L-cysteine, 0.10 g L-phenylalanine, 0.80 g L-threonine, 0.13 g L-tryptophan, 0.15 g L-valine/6.22 g).

† Vitamin mixture.\textsuperscript{22}

‡ Mineral mixture.\textsuperscript{22}

Chol, choline chloride; GB, glycine betaine hydrochloride; Car, carnitine chloride; PC, phosphatidylcholine; TMA, trimethylamine; TMAO, trimethylamine oxide.
TMA). To 1 g of the powder mixture was added 200 µL of distilled water, a trace amount of α-tocopherol, 100 µL of corn oil and 200 µL of the labeled compounds (0.1 mCi/mL ethanol). The mixtures were homogenized by mixing again and samples were air-dried at room temperature (25–27°C).

Another diet supplemented with [15N]-choline was prepared by replacing non-labeled choline in the Chol diet with [15N]-choline.

Administration of N-trimethyl compounds

Non-labeled compounds

Fish were fasted during the period of seawater adaptation except on the final 10th day. That is, administration of the test diets was started simultaneously with achieving seawater adaptation. The diets supplemented with N-trimethyl compounds were given three times a day for 20 days, and fish were killed 48 h after the final feed.

Labeled compounds

Administration of labeled compounds was started on the 10th day, after completion of seawater adaptation. Labeled compounds, including choline ([methyl-14C] and [1,2-14C]) and [14C]-TMA, were administered to tilapia three times a day for 3 days after fasting for 1 day, and fish were killed 48 h after the final feed. For injection studies, substrates were dissolved in distilled water and injected into the intraperitoneal cavity twice a day for 2 days using a 10 µL microsyringe (Hamilton). Twenty-four hours after the final injection, the fish were anesthetized and frozen (~20°C). The feeding test for the diet supplemented with [15N]-choline was carried out in the same way as described for the test with the Chol diet except that the feeding period was 23 days. Each of the four individuals was fed the diet containing 1.8 mmol of [15N]-choline chloride for 23 days. Two days after the final feed, the fish were anesthetized before the muscles were isolating, and these were stored at ~80°C.

In vitro intestinal studies

Fish were dissected on the 10th day after completing seawater adaptation. They were fed the commercial diet (Shikoku-Kumiai) during the rearing period and until the day before they were killed and the intestine and stomach excised.

After removing the intestine, the intestinal contents from four individuals were pooled and mixed well. A small amount of the mixture was placed on a pH Probe (Horiba, Kyoto, Japan) for measurement. Unless otherwise stated, the pH of the media was adjusted to 8.0 because the pH value for intestinal contents obtained was 8.2.

To study TMA formation from N-trimethyl compounds including choline, glycine betaine and carnitine in the digestive tract, solutions for injection were prepared. Physiological saline containing NaCl, KCl, CaCl2, NaHCO3 and MgCl2 at 0.230, 0.008, 0.002, 0.002 and 0.004 mol/L, respectively, was used for dilution of the compounds for seawater fish.23 Substrates for injection were prepared as 0.1 M solutions in physiological saline (Chol, supplemented with choline chloride; GB, supplemented with glycine betaine hydrochloride; Car, supplemented with carnitine chloride). A non-supplemented (Basal) diet was also prepared as a control.

Three types of media were prepared to investigate the effects of penicillin on the formation of TMA from choline in the intestine. Medium containing 2.5% Bacto-tryptone (Difco), 0.60% yeast extract (Difco) and 5.0% choline chloride (pH 8.0) was prepared (+choline). Another medium was also prepared without choline chloride (without addition), but containing 1.0% penicillin G potassium salt (+choline and penicillin). The media components were dissolved in distilled water.

Medium containing [15N]-choline was prepared by replacing non-labeled choline chloride of +choline with [15N]-choline chloride. The medium was injected into the intestine, without adjusting pH due to the small amount used (0.3 mL/g intestine). The removed stomach and intestine were placed on UV-sterilized disposable Petri dishes (depth, 2.0 cm; inner diameter, 8.5 cm; or depth, 1.0 cm; inner diameter, 4.5 cm). UV-sterilized disposable syringes, needles (0.40 mm × 19.00 mm) and filters (pore size, 0.25 µm) were used. The filter was connected between the needle and syringe when the media was injected into the cavity of the stomach and intestine (~0.5 mL/g intestine).

Activity for formation of TMA from TMAO in the fish intestine was evaluated with [14C]-TMAO. The intestines were placed on UV-sterilized disposable Petri dishes (depth, 2.0 cm; inner diameter, 8.5 cm; or depth, 1.0 cm; inner diameter, 4.5 cm). [14C]-TMAO solution (0.025 µCi/µL) was injected without further sterilization (~10 µL/g wet tissue). A 10 µL microsyringe (Hamilton) sterilized with 70% ethanol was used for the injection.
All operations were carried out under sterile conditions. Incubations were carried out at 27°C for 7 h except for the penicillin experiment, which was done at 37°C.

**Incubation of intestinal microorganisms in the syringe**

Fish were excised on the 10th day after completing seawater adaptation. They were fed the commercial diet during the rearing period and until the day before they were killed and their intestine was excised. Three types of media were prepared to investigate the microbial reduction of TMAO to TMA in the intestine. Media containing 1.0% penicillin G (without addition), but containing 1.0% penicillin G and TMAO (with TMAO), and media components were dissolved in artificial seawater prepared with an artificial seawater mix (Marine merit, Matsuda). The pH of the medium was adjusted to 8.5.

The microorganisms were incubated with the use of UV-sterilized disposable syringes (Terumo, Japan), 0.25 μm syringe filters (Advantec Toyo, Tokyo, Japan) and hypodermic needles (Terumo), and autoclave-sterilized silicon rubber. The intestinal tissue was homogenized in a five-fold volume of the filter-sterilized physiological saline using a glass homogenizer (diameter, 2.5 cm; length, 18.5 cm) sterilized with 70% ethanol. A 50 mL syringe was dismantled into the cylinder and piston. Then, 35 mL of the filtrated medium, ‘without addition’, was taken up in the cylinder connected with the hypodermic needle stuck in the silicon rubber. Next, 4 mL of the homogenate as a mixture of intestinal microorganisms was inoculated into the medium and the top of the cylinder was connected to the piston. The sealed syringe was inverted and the head-space air inside the syringe was carefully expelled through the needle. Finally, the syringe was completely sealed by sticking the needle into the rubber again, and preincubated at 27°C for 2 days.

Experimental incubation was carried out using this incubate as a mixture of intestinal microorganisms. First, samples of 25 mL of the three media (without addition, +TMAO, and +TMAO and penicillin) in 30 mL syringes were inoculated with 1 mL of the preincubated media. All of the operations were carried out in the same manner as the preincubation. They were incubated at 27°C for 30 h. During this period, the TMA formed was quantified and turbidity at 660 nm was measured.

**Preparation of tissue extract**

For determination of TMA and TMAO by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS), the tissues (frozen at –80°C) or the incubated media were homogenized with an equal volume of 10% trichloroacetic acid using a Polytron homogenizer (PT 10–35; KINEMATIKA). The homogenate was centrifuged at 10 000 ×g for 20 min and the supernatant was made up to 10 mL with 5% trichloroacetic acid.

In the tracer experiment using the [14C]-labeled substances, a mortar (depth, 10.5 cm; inner diameter, 17 cm) with a pestle (length, 21 cm) was used for homogenization instead of the Polytron. The whole body of the fish was homogenized in 10% trichloroacetic acid. Subsequently, the homogenate was centrifuged at 10 000 ×g for 20 min. The supernatant was recovered and made up to 10 mL with 5% trichloroacetic acid.

The intestines with [15N]-choline injected into the cavity were pooled and homogenized with an equal volume of 10% trichloroacetic acid using the Polytron homogenizer and centrifuged at 10 000 ×g for 20 min. The supernatant was made up to 10 mL. In the case of non-labeled substrates, the trichloroacetic acid concentration was 5% the same as that of the labeled substrates.

The intestines with [3H]-TMAO injected into the cavity were homogenized with an equal volume of 5% perchloric acid using a glass homogenizer and centrifuged at 10 000 ×g for 20 min. The supernatant obtained was used for HPLC estimation of [3H]-TMA. The sample extracts were diluted 10-fold with distilled water before submission to HPLC.

**Gas liquid chromatography analysis for determination of TMA and TMAO**

Trimethylamine and TMAO were determined on a gas liquid chromatograph (GLC-8 A GC; Shimadzu), equipped with a flame ionization detector. A glass column (3 mm × 3 m) was packed with Thermon 3000 (5%), KOH (1%) and Sunpak-A (80–100 mesh). The analytical conditions adopted were as follows: injection port temperature, 250°C; detector temperature, 250°C; column temperature, 100°C; the flow rate of carrier gases for both N₂ and H₂ was 50 mL/min. Shimadzu Chromatopack C-R3A was used as an integrator.

Samples of 1.0 or 2.0 mL of trichloroacetic acid extracts were placed in Pierce vials with a bored screw-top fitted with a sheet of silicone rubber (volume, 3.5 mL; height, 4.5 cm; diameter, 1.5 cm). To these extracts was added 0.5 mL of diethyl ether.
and 1 mL of 65% KOH. The mixture was shaken well for 30 s and then kept at 50°C for 3 min in a water bath. After vigorous shaking for 1 min, the mixture was left at room temperature for 10 min to allow the phases to separate. The diethyl ether layer (4 μL) was applied to the GC column for analysis. For determination of TMAO, TMA was determined by the method described previously, after TMAO in the tissue extracts was reduced to TMA by adding a drop of 10% TiCl₃. Trimethylamine oxide levels were calculated from the difference between the amounts of TMA obtained with and without TiCl₃.

Analysis of [¹⁵N]-TMA and [¹⁵N]-TMAO

A QP5050A gas chromatography-mass spectrometry (GC-MS; Shimadzu) equipped with a CI-50 chemical ionization system (Shimadzu) and a capillary column DB-5 (60 m × 0.25 mm; J & W Scientific Inc.) was used to detect [¹⁵N]-TMA. The analytical conditions were as follows: injection port temperature, 260°C; initial column temperature, 40°C (5 min); rate of temperature increase, 5°C/min; final column temperature, 240°C (15 min); detector voltage, 1.6 kV.

Authentic 0.1 M TMA solutions (non-labeled and [¹⁵N]-labeled) and their binary mixture obtained by mixing equal volumes of the solutions (binary mixture) were prepared. Each solution (0.2 mL) was put into a 14 mL pierce vial with 3.8 mL of distilled water, 4 mL of 5% trichloroacetic acid and 4 mL of 65% KOH. After shaking well, 1 mL of head space gas was injected into a GC-MS analyzer with a 1 mL disposable syringe.

In the case of the sample solution, 2 mL samples from 10 mL of muscle extract (1 g wet tissue/10 mL 5% trichloroacetic acid) of each of four individuals were placed together in a pierce vial (volume, 14 mL; height, 7 cm; diameter, 2.0 cm) and 10% TiCl₃ was added drop-wise and kept at room temperature (25°C) for more than 2 h. Then, 4 mL of 65% KOH was added to the reaction mixture (total volume, about 12 mL), shaken well and 1 mL of the headspace gas was injected into the GC-MS with the syringe. The amount of TMAO was evaluated from the difference between the amounts of TMA obtained with and without TiCl₃ as described above. Background subtraction was done for each of the TMA peaks to obtain final MS spectra.

Preparation of crude enzymatic solution

Fish were excised on the 10th day after completing seawater adaptation. They were fed a commercial diet during the rearing period and until the day before they were killed and their tissues were excised.

To measure enzymatic activity of TMA monoxygenase and formation activity of TMA from choline, tissues were excised from the fish, frozen with liquid nitrogen, and transferred immediately to a −80°C freezer cabinet. Each tissue, such as, liver, kidney, gill, muscle, brain, intestine, stomach and eye was homogenized in five volumes of ice-cold 0.25 M sucrose (pH 7.8) dissolved in 0.1 M Tris buffer using a glass homogenizer (diameter, 2.5 cm; length, 18.5 cm).

Trimethylamine-forming activity from [methyl-¹⁴C]-choline

[Methyl-¹⁴C]-choline chloride (10 μL, 1.5 × 10⁻³ μCi/μL) was placed in a 1.5 mL test tube and 100 μL of each crude enzymatic solution was added, mixed and incubated at 27°C for 3 h. Distilled water (390 μL) and 5% perchloric acid (500 μL) were added and the mixtures were centrifuged at 3500 × g for 10 min. The supernatant (0.5 mL) of the mixture was transferred to a pierce vial with silicone rubber inside the cap (volume, 3.5 mL; height, 4.5 cm; diameter, 1.5 cm), and 1.0 mL of toluene and 1.0 mL of 65% KOH were added. The mixture was shaken well and centrifuged at 3500 × g for 10 min. The toluene layer (0.5 mL) was used for measurement of radioactivity with 5 mL of scintillation cocktail.

Fish were dissected on the 10th day after completing the seawater adaptation. They were fed a commercial diet during the rearing period and until the day before sacrifice and excision of the tissues. The removed stomach and intestine were placed on UV-sterilized disposable Petri dishes (depth, 2.0 cm; inner diameter, 5.5 cm; or depth, 1.0 cm; inner diameter, 4.5 cm) under sterile conditions. Then, 100 μL of [methyl-¹⁴C]-choline chloride (1.5 × 10⁻³ μCi/μL in water) was introduced into these tissues (~1 g wet weight) using UV-sterilized disposable syringes (1 mL), needles (0.40 × 19 mm) and filters (0.25 μm). The filter was connected between the needle and syringe when the media was injected into the cavity. After 3 h of incubation at 27°C, each specimen was homogenized with an equal volume of 5% perchloric acid using a glass homogenizer (diameter, 2.5 cm; length, 18.5 cm) and centrifuged at 3500 × g for 10 min. The supernatant (500 μL) was transferred to a vial, and 1 mL of toluene and 1 mL of 65% KOH were added, shaken well and the mixture was centrifuged at 3500 × g for 10 min. The toluene layer
(0.2 mL) was used to measure radioactivity in the same manner as described previously.

**Trimethylamine monoxygenase assay**

The crude tissue extract (100 μL), 10 μL [14C]-TMA chloride (0.04 μCi/μL), 13 nmol FAD, 12.8 μmol MgCl₂, 0.35 μmol NADPH, and 40 μmol potassium pyrophosphate buffer (pH 8.2), were mixed and made up to 500 μL as a reaction mixture by adding distilled water. The reaction was started with the addition of [14C]-TMA at 27°C and stopped by adding 500 μL of 5% perchloric acid after 30 min. After centrifugation at 3500 × g for 10 min, 0.5 mL of the supernatant was used to isolate [14C]-TMAO according to the method described by Baker and Chaykin.19,20 Aliquots of the supernatant were applied to cation-exchange columns containing 500 mg of Dowex 50 W X12 (200–400 mesh, H⁺ form, Dowex). Neutral molecules were washed out of the column by 4 mL of water. The [14C]-TMAO retained was eluted with 2 mL of 5% aqueous ammonia. The eluate was dried under an infrared lamp (250 W). The residue obtained by drying was dissolved in 5 mL of the liquid scintillation cocktail to measure radioactivity.

**Statistical analysis**

Data are presented as mean ± SD unless otherwise stated. Differences between two groups were evaluated by the Student’s t-test.

**RESULTS**

**Precursor of trimethylamine oxide**

The distribution of TMAO in tissues (liver, kidney, eye, muscle, brain, gill and intestine) is shown in Table 2. Most of the TMAO was exclusively localized in the muscle. The eye and gill contained much smaller amounts, and no TMAO was detected in other tissues. No TMA was detected in any of the tissues examined (Table 2). Fish were fed each diet supplemented with TMAO, TMA, choline, glycine betaine, carnitine or PC, and the effects on TMAO concentration in the muscle were investigated. Compared with the Basal diet, the TMAO diet was the most effective for accumulation of TMAO (P<0.005) followed by the TMA diet (P<0.005). Among the quaternary ammonium compounds tested for TMAO accumulation including PC as a derivative of choline (Fig. 1), the Chol diet was the most effective (P<0.005), followed by the PC diet (P<0.01). The GB and Car diets were not effective for TMAO accumulation (Fig. 1). Incorporation of radioactivity into TMAO by administration of ([methyl-14C] and [1,2-14C])-choline and [14C]-TMA (as a positive control) was evaluated (Table 3). Radioactivity was incorporated into TMAO at a higher level from [14C]-TMA than from [methyl-14C]-choline. About 0.25% (dietary [methyl-14C]-choline), 2.5% (dietary [14C]-TMA) and 4.1% (injected [14C]-TMA) of each activity was incorporated into TMAO. However, no incorporation of radioactivity was detected from [1,2-14C]-choline (Table 3). Both TMAO and choline have a trimethyl ammonium group. Therefore, it is possible that the trimethyl ammonium group of choline is utilized for synthesis of TMAO in the fish. We have already shown that the methyl-carbon of choline was incorporated into TMAO (Table 3). The possibility of incorporation of the nitrogen atom of choline into TMAO was therefore investigated. The TMAO formed in the muscle of tilapia after administration of the diets supplemented with non-labeled and [15N]-labeled choline was analyzed. The retention time of TMA on GC was 3.9 min. Non-labeled TMA had a fragmentation pattern of m/z 59 (M⁺), 58, and 42. [15N]-TMA had a pattern of 60 (M⁺), 59 and 43. When combined, all fragments were observed (60, 59, 58, 43 and 42). These spectra were utilized as references to identify non-labeled and [15N]-labeled TMA. When the muscle extracts were reduced by TiCl₃, TMA was detected after 3.9 min. Fragmentation patterns indicated m/z 59, 58 and 42 for non-labeled TMA and 60, 59, 58, 43 and 42 for [15N]-labeled TMA. Both patterns were the same as those of the authentic non-labeled TMA and binary mixture.

**Trimethylamine formation from choline in the intestine**

Quaternary ammonium compounds (choline, glycine betaine and carnitine) were introduced into

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Average amount × 10⁻² (mmol/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>ND</td>
</tr>
<tr>
<td>Kidney</td>
<td>ND</td>
</tr>
<tr>
<td>Eye</td>
<td>5</td>
</tr>
<tr>
<td>Muscle</td>
<td>74 ± 28</td>
</tr>
<tr>
<td>Brain</td>
<td>ND</td>
</tr>
<tr>
<td>Gill</td>
<td>16</td>
</tr>
<tr>
<td>Intestine</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not detected; TMAO, trimethylamine oxide. n = 6.
the isolated stomach and intestine (Fig. 2). Trimethylamine formation was detected only in the intestine of fish that received choline. However, it was not detected in the tissue of fish receiving glycine betaine or carnitine (appreciable amounts of TMA were detected in the intestine, i.e. less than 0.6 mmol/100 g; Basal, GB and Car groups). Trimethylamine was only detected in the stomach of the Car group (Fig. 2). Tissue homogenates prepared from the liver, kidney, eye, muscle, brain, gill, stomach and intestine were incubated with [methyl-14C]-choline, and TMA-forming activity was evaluated (Table 4). After a 3 h incubation, no TMA formation was observed in any tissue examined. However, when medium supplemented with [methyl-14C]-choline was introduced into the cavity of the whole stomach or intestine, an appreciable amount of [14C]-TMA was detected in the intestine.

Medium supplemented with [15N]-choline was introduced into the whole intestine and incubated at 27°C for 7 h. To the mixture was added 65% KOH, and the headspace gas was analyzed by GC-MS for TMA. The spectrum of the peak at 3.9 min showed a pattern similar to that of authentic [15N]-TMA. Figure 3 shows the effects of penicillin on the formation of TMA from choline in the isolated intestine after incubation at 37°C for 7 h. Trimethylamine was increased in the intestine when +choline was introduced into the intestine (P < 0.005). However, the formation of TMA was clearly suppressed in the intestine treated with medium supplemented with choline together
with penicillin (+choline and penicillin; \( P < 0.005 \); Fig. 3).

**Trimethylamine formation from trimethylamine oxide in the intestine**

We could detect \( ^{14}\text{C} \)-TMA formation in the intestine by intestinal injection of the aqueous \( ^{14}\text{C} \)-TMAO solution after 7 h incubation in either of two individuals (Table 5).

Microbial growth and TMA in the media were measured during the incubation of intestinal microorganisms in a closed syringe to prevent supply of oxygen (Fig. 4). The formation of TMA was observed in medium supplemented with TMAO (+TMAO). Penicillin completely suppressed the formation of TMA. In the basal medium (Basal) and +TMAO, the turbidity at 660 nm were elevated with time in contrast to the +TMAO and penicillin medium. This showed that growth of microorganisms was markedly suppressed by the addition of penicillin (Fig. 4).

**Monooxygenation of trimethylamine to trimethylamine oxide**

Figure 5 shows the distribution of TMA monooxygenase activity among various fish tissues. Of all the tissues tested, the highest activity was detected in the liver followed by the kidney. The activity was decreased to about 20% in the absence of NADPH compared with that in the presence of NADPH (Fig. 5).

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**Table 3** Incorporation of radioactivity into TMAO formed after administration of \( ^{14}\text{C} \)-labeled choline and TMA to seawater-adapted tilapia

<table>
<thead>
<tr>
<th>Method</th>
<th>Labeled substrate</th>
<th>Total dose during the experimental period (( \mu \text{Ci/fish} ))</th>
<th>TMAO recovered in PCA-soluble fraction (( \mu \text{Ci/fish} ) ( \times ) 10(^5 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>[1.2,(^{14}\text{C})]-choline</td>
<td>0.68</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>[Methyl-(^{14}\text{C})]-choline</td>
<td>0.68</td>
<td>170 ( \pm ) 200</td>
</tr>
<tr>
<td></td>
<td>(^{14}\text{C})-TMA</td>
<td>0.68</td>
<td>1700 ( \pm ) 800</td>
</tr>
<tr>
<td>Injection</td>
<td>[1.2,(^{14}\text{C})]-choline</td>
<td>0.29</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>[Methyl-(^{14}\text{C})]-choline</td>
<td>0.29</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>(^{14}\text{C})-TMA</td>
<td>0.29</td>
<td>1200 ( \pm ) 300</td>
</tr>
</tbody>
</table>

ND, not detected; PCA, perchloric acid; TMA, trimethylamine; TMAO, trimethylamine oxide. \( n = 3 \).

**Table 4** Distribution of TMA-forming activity from \( ^{[\text{methyl-14C]} \) choline in the tissues of seawater-adapted tilapia

<table>
<thead>
<tr>
<th>Individuals</th>
<th>Ratio of ( \mu \text{Ci}/(\text{toluene fraction/total/ h}) \times 10^5 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue homogenate</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>ND</td>
</tr>
<tr>
<td>Kidney</td>
<td>ND</td>
</tr>
<tr>
<td>Eye</td>
<td>ND</td>
</tr>
<tr>
<td>Muscle</td>
<td>ND</td>
</tr>
<tr>
<td>Brain</td>
<td>ND</td>
</tr>
<tr>
<td>Gill</td>
<td>ND</td>
</tr>
<tr>
<td>Stomach</td>
<td>ND</td>
</tr>
<tr>
<td>Intestine</td>
<td>ND</td>
</tr>
<tr>
<td>Whole tissue</td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>6</td>
</tr>
<tr>
<td>Intestine</td>
<td>300</td>
</tr>
</tbody>
</table>

ND, not detected.

---

**Fig. 3** Effects of penicillin on the suppression of trimethylamine formation from choline. *Significant difference compared to +choline (\( P < 0.005 \)). Error bars represent SD of means (\( n = 3 \)).
Biosynthesis of TMAO in seawater tilapia

**DISCUSSION**

Most TMAO was found to be localized exclusively in the muscle (Table 2). The muscle therefore can be a useful index for the accumulation of TMAO. There was a much greater elevation of TMAO content in the muscle when the fish were fed the diet supplemented with TMAO. These results indicated that the muscle is the most important tissue for storage of TMAO, and that addition of exogenous TMAO effectively produced accumulation of TMAO. Trimethylamine oxide may be directly incorporated from the diet into fish tissues. In teleosts, such as, Chinook salmon *Oncorhynchus tschawytscha*;25 grass puffer *Takifugu niphobles* and filefish *Monacanthus cirrhifer*,26 high levels of TMAO occurred after they were fed a diet supplemented with TMAO. Trimethylamine oxide can be readily accumulated under higher osmotic pressure where excretion of urine is largely restricted.2,27 Trimethylamine oxide concentration in the muscle was 0.74 ± 0.28 mmol/100 g in Nile tilapia (Table 2), much less than the concentrations of 5–10 mmol/100 g reported in squid or 10–20 mmol/100 g reported in elasmobranchs. The diet supplemented with TMA induced accumulation of TMAO in the muscle of tilapia as reported in chum salmon *Oncorhynchus keta* by Daikoku *et al*.28 In tilapia, TMA was not detected in tissues, suggesting that much of the dietary TMA was oxygenated immediately after absorption in the intestine. The residual TMA not absorbed via the intestine was considered to be directly excreted in the feces. Trimethylamine is probably the direct precursor of TMAO in tilapia. In marine fish, TMAO is readily available in the diet and accumulates in the body. Some of the TMAO taken up by the fish might be directly incorporated into the body and residual TMAO might be reduced to TMA in the intestine then absorbed because we observed TMAO to TMA reduction activity in intestine and by intestinal microorganisms under microaerobic conditions (Table 5; Fig. 2). Sakata *et al.* reported that there are significant numbers of anaerobes in the intestine of tilapia.29 But, in our experiments, the microbial flora might be changed during the seawater adaptation. There, more or less, is a possibility that some anaerobes perform the reduction reaction to get energy. In the intestine, TMAO might be the terminal electron acceptor and thus be reduced immediately to TMA as follows:2

\[
\text{TMAO} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{TMA} + \text{H}_2\text{O}
\]

The accumulation of TMAO occurred in the muscle after administration of several quaternary ammonium compounds. Trimethylamine oxide levels in

---

**Table 5**  
<table>
<thead>
<tr>
<th>Individuals</th>
<th>Weight of tissues (g)</th>
<th>TMAO injected (μCi)</th>
<th>Total activity recovered (μCi/g wet tissue)</th>
<th>TMAO recovered (μCi/g wet tissue)</th>
<th>TMAO recovered (μCi/g wet tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.6</td>
<td>0.75</td>
<td>0.22</td>
<td>ND</td>
<td>0.19</td>
</tr>
<tr>
<td>B</td>
<td>5.9</td>
<td>1.7</td>
<td>0.23</td>
<td>ND</td>
<td>0.18</td>
</tr>
</tbody>
</table>

*Radioactivity was measured by liquid scintillation counting. TMA, trimethylamine; TMAO, trimethylamine oxide.
the muscle were markedly elevated by feeding with the diet supplemented with choline, Chol (Fig. 1). Trimethylamine might be formed gradually from choline in the intestine (Fig. 2). In humans, rats and poultry, dietary choline has been shown to be degraded to TMA and oxygenated to TMAO in the liver. Trimethylamine as the direct precursor of TMAO has been demonstrated in marine animals mostly by injection of radioactive substrates as in the starry flounder Platichthys stellatus and nurse shark Ginglymostoma cirratum. The copepods Calanus finmarchicus and C. hyperboreus produce TMAO from TMA. Plankton therefore may synthesize and accumulate TMAO, which may be incorporated into the bodies of fish that consume these species of plankton (i.e. crustaceans). Humans, rats and poultry also produce TMAO from TMA, and it has been detected in urine, sweat and/or eggs. Our experimental data from tilapia were consistent with these reports. That is, muscular TMAO content was increased by administration of TMA (Fig. 1) and radioactive TMAO was formed by dietary administration and intraperitoneal injection of [14C]-TMA (Table 3). These results indicated that TMAO is formed by oxidation of TMA in these animals.

In previous studies on the starry flounder, when [methyl-14C]-choline was administered by intraperitoneal, intramuscular or intravenous injection, detectable radioactive TMAO was recovered only by intraperitoneal injection of [methyl-14C]-choline, suggesting that choline is a precursor of TMAO. In contrast, there was no detectable response in the teleost cod after intraperitoneal injection of [methyl-14C]-choline. Bilinski further reported that radioactivities of [methyl-14C]-γ-butyrobetaine (starry flounder), [methyl-14C]-glycine betaine (lemon sole Parophrys vetulus) and [methyl-14C]-L-methionine (lemon sole; American lobster) were also incorporated into TMAO. However, the intraperitoneal injection of [methyl-14C] and [1,2-14C]-choline did not induce the formation of radioactive TMAO in tilapia (Table 3). Radioactive TMAO was observed after dietary administration of [methyl-14C]-choline. Trimethylamine oxide recovered from animals fed [1,2-14C]-choline was not radioactive. In our experiments in tilapia, the methyl group of choline was incorporated into TMAO only in the case of oral administration but not after intraperitoneal injection. The methyl group metabolized in the intestine may be utilized to form TMAO.

To confirm the incorporation of the trimethyl ammonium group of choline into TMAO, incorporation of the nitrogen atom of choline labeled with the stable isotope 15N. When the diet supplemented with [15N]-choline was administered, incorporation of 15N into TMAO was observed in the muscle of fish. The fragmentation pattern of TMAO was consistent with the presence of [15N]-TMAO. The fragments of m/z 58 (non-labeled TMA) and 59 ([15N]-TMA) could be formed from [(CH₃)₂N⁺CH₂] produced by α-elimination (M⁺ – 1). The fragment of m/z 42 (non-labeled TMA) and 43 ([15N]-TMA) could be formed from [CH₂N = CH₃]⁺ after intramolecular β-elimination of the former ion fragment (M⁺ – H – CH₃).

Marked differences were observed in [14C]-TMAO levels depending on the method of administration of [methyl-14C]-choline (Table 3). Choline was degraded to TMA in the intestine (Fig. 2) and the formation of TMA from choline in the intestine
was suppressed by addition of penicillin (Fig. 3). These observations indicated that gut microflora metabolize choline to TMA.

As shown in Table 4, the methyl group of [methyl-15N]-choline was effectively incorporated into TMA in the recovered intestine. When [15N]-choline was injected into the recovered intestine, MS spectra for TMA showed m/z 60 as the molecular ion and the MS chromatogram showed a high peak of m/s 60. These observations strongly suggested that the 15N atom of [15N]-choline was incorporated into the TMA thus formed and that the (CH3)3N+ group of choline was directly transformed to TMA in the intestine.

The formation of TMA in the intestinal homogenate was not observed under aerobic conditions (data not shown). This observation and the inhibition by penicillin suggested that anaerobic intestinal microorganisms were able to form TMA from choline. Trimethylamine was shown previously to be formed from choline37–40 and glycine betaine.41 Some soil bacteria (i.e. Vibrio cholinericus N. SP) are able to stoichiometrically degrade choline into TMA, with acetaldehyde as an intermediate compound.42,43 The marine isolate Sporomusa degrades glycine betaine to TMA, acetate, carbon dioxide and N,N-dimethylglycine.41 These reactions have their own significance for the survival of microorganisms but TMA thus formed is so stable that with the exception of the bacteria (Methylotroph sp.); it can be neither decomposed nor used as a nitrogen and/or carbon source. These data support the presence of anaerobic bacteria in the intestine of fish and indicate they are responsible for TMA formation from choline.

Our results indicated that TMA was not oxidized in the intestine but appeared to be absorbed and transferred to the liver and kidney where TMA monoxygenase (EC.1.14.13.8) was located (Fig. 5). Oxygenation proceeded as follows:

\[(CH_3)_3N + NADPH + H^+ + O_2 \rightarrow (CH_3)_3NO + NADP^+ + H_2O.\]

The enzyme occurs in a wide range of species from microorganisms to mammals.44–46 In microorganisms, TMA monoxygenase activity has been detected in Pseudomonas aminovorans and the enzyme has been partially purified and characterized.21 The enzyme activity has been reported in several teleosts, such as, the eel Anguilla japonica and guppy Poecilia reticulata,47 cod Gadus morhua5 and turbot Scophthalmus maximus,48 and cartilaginous nurse shark Ginglymostoma cirratum.33 However, little activity was detected in the incubation media of digestive tissues (Figs 2, 3; Table 4) probably because there was no supply of oxygen as a substrate or no microorganisms possessing the enzyme were present. We observed TMAO to TMA reduction activity in intestinal microorganisms under microaerobic conditions (Fig. 4). In the present study, TMA monoxygenase activity was detected in the liver, kidney, eye, muscle, gill and intestine but not in the brain (Fig. 5). The reaction is commonly carried out by flavin-containing monoxygenase. It mediates not only N-oxidation of TMA but also other N- and S-oxidations.46 In contrast, Larsen and Schlenk27 reported that rainbow trout Oncorhynchus mykiss exposed to 21‰ seawater for 48 h exhibited flavin-containing monoxygenase activity in the gill and gut (determined by oxidation of thiourea but not TMA). In contrast to our results, the activity in the gill and gut was higher than that in the liver. This is because Larsen and Schlenk27 evaluated the activity based on the amount of microsomal protein, while it was simply estimated based on the weight of wet tissue in the present study (Fig. 5). The total amounts of microsomal protein of gill or gut per wet tissue may be smaller than those of the liver or kidney. In addition, the amount of monoxygenase protein per total amount of microsomal protein in the gill and gut might be higher than those in the liver. This might allow the gill and gut to exhibit higher activity than the liver only when expressed per milligram of microsomal protein. In addition, we did not remove intestinal contents, while this was done in the previous study in trout. Most of the TMAO retained in the intestine would immediately be reduced to TMA; in fact we detected the activity of reducing TMAO in the intestinal microorganisms (Fig. 4). Therefore there was little contribution of the intestine to monoxygenation in tilapia.

Larsen and Schlenk27 showed that plasma osmolarity in rainbow trout was increased by salinity treatment. Correspondingly, flavin-containing monoxygenase activity, its protein expression and concentrations of TMAO with urea were increased by the salinity treatment. Therefore, in rainbow trout, TMAO is regarded as the compound related to salinity stress although it is not a ‘compatible solute’. Trimethylamine oxide can force thermodynamically unfolded proteins to fold49,50 and counteract the urea perturbation of protein function.3 Urea levels in teleost fish, especially under freshwater conditions, are commonly much lower than those in elasmobranch fish in seawater. Larsen and Schlenk reported that only small amounts of TMAO and urea were detected in the tissues (muscle, liver, gill, kidney, gut and plasma) of the trout Oncorhynchus mykiss under freshwater conditions.27 It is interesting that flavin-containing monoxygenase, TMAO and urea levels were elevated by salinity treatment. Greater accumulation
of urea in fish tissue by salinity is associated with greater contribution of TMAO to stabilization of functional proteins. Similar phenomena might be expected in the euryhaline teleost, tilapia.

We have already undertaken the same experiments using freshwater reared tilapia. Similar results to these seawater reared tilapia were obtained. Anthoni et al. reported that freshwater tilapia had strong systems for both TMAO synthesis and TMAO accumulation just like the seawater reared tilapia presented in this paper. High adaptability of tilapia to seawater conditions might be, at least partly, dependent on these TMAO related systems.

In summary, the results of the present study indicate the mechanism responsible for TMAO accumulation induced by feeding a diet containing choline. First, TMA is microbiologically formed from dietary choline in the intestine, and the TMA is then absorbed and converted into its N-oxide form, TMAO, in the liver and/or kidney. Accumulation of TMAO induced by feeding a diet containing choline seemed to occur in marine fish. Trimethylamine oxide may be synthesized through this process and accumulate mainly in the muscle in these fish. Trimethylamine oxide is readily produced from dietary choline and then stored mainly in the muscle even if the diet is free of both TMA and TMAO. It is worth noting that both the intestinal microorganisms and the tissue oxygenase are indispensable for constructing the TMAO pool in fish muscle. In addition, dietary TMAO might be indirectly transferred to the muscle via microbial reduction to TMA.

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