Maintenance and accumulation of trimethylamine oxide by winter skate (Leucoraja ocellata): reliance on low whole animal losses rather than synthesis

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Triamethylamine oxide (TMAO) is typically accumulated as an osmolyte in marine elasmobranchs to levels second only to urea. Winter skates maintain plasma TMAO levels for up to 45 days without feeding. The liver displays methimazole oxidation, which is consistent with the presence of flavin-containing monooxygenase (E.C. 1.14.13.8) activity, the class of enzymes responsible for the physiological oxygenation of trimethylamine (TMA) to TMAO in mammals. Winter skates maintain plasma TMAO levels for up to 45 days without feeding. The liver displays methimazole oxidation, which is consistent with the presence of flavin-containing monooxygenase (E.C. 1.14.13.8) activity, the class of enzymes responsible for the physiological oxygenation of trimethylamine (TMA) to TMAO in mammals. However, no evidence for TMA oxygenation by winter skates was found using in vivo or in vitro techniques, indicating no significant capacity for endogenous TMAO synthesis. Fed skates displayed low, but measurable (1–13 μmol·kg⁻¹·h⁻¹), efflux of TMAO (plus TMA), whereas fasted skates did not. Using the loss of injected [¹⁴C]TMAO, it was determined that whole animal TMAO losses are likely <1% of whole body TMAO per day. These results demonstrate that winter skates utilize low whole animal TMAO losses, rather than endogenous synthesis, to maintain TMAO levels when not feeding.

elasmobranch; excretion; flavin-containing monooxygenase; trimethylamine oxide synthesis

TRIMETHYLAMINE OXIDE (TMAO) is a small nitrogenous compound that is commonly accumulated to levels of 35–180 mM in marine elasmobranchs (sharks, skates, and rays) as part of the osmoconforming osmoregulatory strategy (32). Among the organic solutes accumulated by marine elasmobranchs, TMAO concentrations are second only to urea, which is the predominant osmolyte at ~350–450 mM (reviewed in Ref. 32). The primary role of TMAO in elasmobranchs is that of an osmolyte, with levels varying in response to osmotic challenge in the extracellular and intracellular fluid (6, 15, 22). Nonosmolyte roles of TMAO have also been hypothesized, including the counteraction of deleterious effects high urea concentration (31, 33) and hydrostatic pressure (11, 33) have on macromolecular structure, as well as contributing to buoyancy (28). There has also been speculation that TMAO may be a significant component of total nitrogen excretion (29). Despite the apparently ubiquitous accumulation of TMAO in marine species and the diverse roles of this compound in elasmobranchs, the regulation and metabolism of TMAO have received relatively little detailed study. In addition, many of the available data on whole animal TMAO regulation in marine elasmobranchs are either contradictory or have been more recently challenged, resulting in fundamental gaps in the current understanding of this compound.

The spiny dogfish (Squalus acanthias) and little skate (Leucoraja erinacea) are the most extensively studied elasmobranchs with respect to the regulation, retention, and metabolism of TMAO. Neither species has detectable capacity for the oxygenation of trimethylamine (TMA) to TMAO (2, 7, 9), which is the only known synthesis pathway for TMAO in vertebrates; however, at least in the case of spiny dogfish, plasma concentrations of TMAO can be maintained for several weeks without feeding (4, 9, 23). The capacity to maintain plasma levels for prolonged periods without an exogenous supply of TMAO has been partially attributed to the reabsorption of more than 90% of the TMAO filtered by the kidney, (4) as well as to the release of TMAO from the muscle (9). The little skate also reabsorbs filtered TMAO in the kidney (8), although the capacity to maintain TMAO without feeding has not been examined in the little skate.

The apparent lack of endogenous TMAO synthesis in spiny dogfish, and by extension little skate, has been challenged by Schlenk (18). In mammals, the enzyme responsible for the physiological oxidation of TMA in vivo has been identified as flavin-containing monooxygenase-3 (FMO3). The FMOs (E.C. 1.14.13.8) are a class of enzymes (five different FMOs have been identified to date) that are localized in the microsomal fraction, require NADPH as a cofactor, and display broad substrate preference and affinity for a wide range of nitrogen and sulfur-containing organic compounds (3). In general, the primary role attributed to the FMO class of enzymes in vertebrates is the oxidation and detoxification of many nitrogen and sulfur-containing organic compounds (34). Attempts to measure TMAO oxygenation often use crude tissue homogenates as well as to the release of TMAO from the muscle (9). The little skate also reabsorbs filtered TMAO in the kidney (8), although the capacity to maintain TMAO without feeding has not been examined in the little skate.

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The only data on the whole animal loss of TMAO in elasmobranchs are the study of Goldstein and Palatt (10), in which a loss coefficient for radiolabeled TMAO—equivalent to the proportion of whole animal TMAO lost per day—was determined in four species. This often-cited study (for example, see recent reviews 20, 29) found that the loss coefficient for TMAO ranged from 4 to 14% per day depending on the species (10). Whole animal TMAO efflux rates of this magnitude would make it impossible to maintain levels of TMAO for prolonged periods without feeding in elasmobranch species that lack endogenous synthesis. For example, the loss coefficients were calculated to be 10.4 and 7.8% per day for S. acanthias and L. erinacea, respectively; or a loss of ~50 and 40% of the total body TMAO pool, respectively, after 1 wk of food deprivation. There is no evidence for such precipitous declines in TMAO content for either species. Thus either 1) the data indicating a lack of synthesis are erroneous, 2) the determined rate of TMAO loss in elasmobranchs is an overestimate, or 3) both of these aspects of TMAO metabolism are in need of reassessment.

The present study was undertaken to determine which of the above cases is correct. For this, we use the winter skate (L. ocellata), a species that we have recently reported also lacks detectable oxygenation of TMA in the liver (25), using the methodology criticized by Schlenk (18). A series of experiments were designed to determine whether winter skates could maintain circulating TMAO concentrations without feeding and subsequently to elucidate whether this was due to endogenous synthesis, low excretion rates, or a combination thereof. By the nature of these experiments, a number of other aspects of TMAO accumulation were also assessed, including the contribution of TMAO (plus TMA) to whole animal nitrogen excretion, the relationship between extracellular TMAO concentration, and the accumulation of intracellular TMAO (which is indicative of an active uptake mechanism) and the relative permeability of several tissues to TMAO.

MATERIALS AND METHODS

Animals

Winter skates, L. ocellata, were caught by divers in Conception Bay, Newfoundland, and transported to the Ocean Sciences Centre in an aerated live-well. At the Ocean Sciences Centre, skates were held in flow-through seawater tanks on a natural photoperiod at either ambient seawater temperature (4–14°C) or maintained between 7° and 9°C. Animals that were at temperatures other than 7–9°C were acclimated to this temperature for at least 2 wk before being used in the experiments. Fish were fed chopped frozen herring containing 3 or 4 times per week.

Experimental

Effects of food deprivation on plasma TMAO, urea, and osmolality. Relatively large skates were selected (~1.5–4 kg) for this experiment to allow for serial sampling of blood for an extended duration of several weeks. Animals were fed every 3–4 days to satiation for at least 4 wk before starting the experiment. On the first day of the experiment, 3 days after the last feeding, fish were anesthetized by placing them in a seawater bath containing 0.01 ml of eugenol per liter of seawater (stock solution of 10% eugenol in ethanol diluted into seawater). A small blood sample (~1.0 ml) was taken by caudal puncture with a 23-gauge heparinized syringe, and the fish was immediately returned to the tank for recovery. This procedure resulted in zero mortality over the duration of the experiment. Food was withheld, and skates were serially sampled every 7 or 14 days for 6 wk.

Investigations on endogenous synthesis of TMAO. First, in vivo metabolism of injected TMA fasted skates (6 days) were anesthetized with eugenol, as described above, and injected intraperitoneally with [14C]TMAO in 50 mM HEPES buffer (pH 6.5 at 20°C) at a dose of 50 μCi per kg. The activity of the solution was 10.0 μCi/ml, with an injection dose of 0.5 ml/100 g of fish. Animals were then transferred to a second tank of aerated filtered-seawater (1 μm, UV sterilized) for ~20 min to wash off residual eugenol. Following this brief recovery period, skates were put in small individual aquaria with 19 volumes of filtered seawater (1 μm, UV sterilized). Aquaria were partially immersed in water within a blacked out insulated cooler attached to a thermostated chilling unit that maintained the aquaria at 8°C. Each aquarium had two small holes in the lid, one for an air-stone and the other to facilitate water sampling. Water samples were taken over time and analyzed for radioactivity due to the presence of TMA and TMAO by scintillation counting as described below. After 24 h postinjection, animals were anesthetized with eugenol and killed by pithing. Tissue samples were collected and frozen with liquid nitrogen for later analysis (stored at ~65°C).

Second, for in vitro studies, the activity of FMO and TMAO oxygenation was assessed on subcellular fractions collected using methodology adapted from Schlenk and Li-Schlenk (19). Skates were killed by a blow to the head, and liver tissue was removed, blotted dry with paper towel, and frozen with liquid nitrogen for later analysis. Frozen tissue was weighed, diced, and transferred to a glass homogenizer along with 4–6 volumes of ice-cold homogenization buffer. The homogenization buffer consisted of 100 mM Tris-acetate, 100 mM KCl, and 1.0 mM disodium-EDTA (pH 7.4 at 20°C). Tissues were homogenized on ice by two passes of a loose-fitting and two passes of a tight-fitting motor-driven Teflon pestle. The homogenate was centrifuged at 20,000 g for 20 min at 2–4°C to remove heavier subcellular components. The supernant was collected, taking care not to collect any of the floating lipid layer, and centrifuged at 48,000 g at 2–4°C for 45 min to produce a crude microsomal pellet. The pellet was resuspended in a small volume (1–2 ml) of phosphate buffer [100 mM potassium phosphate, 1 mM EDTA, 20% (vol/vol) glycerol, pH 7.4 at 20°C] and frozen in liquid nitrogen for later enzyme analysis. Aliquots of the initial 20,000 g and final 48,000 g supernatants were also collected and frozen to determine the relative enrichment of the final pellet.

Whole animal excretion experiments. To measure whole animal losses of urea, ammonia, and TMAO, skates were placed in individual plastic aquaria with nine volumes of well-aerated filtered seawater (1 μm, UV sterilized). The sides of the aquaria were blacked out, and the lid was opaque with two holes cut in it; one for an air line and one for sampling aliquots of seawater. Temperature was maintained at 7–9°C by storing aquaria in a temperature-controlled cold room.

Initial exploratory experiments were conducted on fed skates, 48 h postfeeding, and on fish that had been fasted for 6 days (complete gut clearance for winter skates is about 4–6 days, J. R. Treberg unpublished observation). Water samples were taken periodically for 24 h after transfer of animals to the aquaria. These experiments indicated that 18 h after transfer was suitable for measuring whole animal losses of urea, ammonia, and TMAO. Seawater samples taken 24 h after skates had been removed from the aquaria were not different from the final sample in the time series, indicating bacterial action had minimal effect on measured variables.

To determine the effects of a single feeding event on whole animal excretory losses, skates were weighed (100 to 360 g), moved to individual 1 m³ seawater tanks (7–9°C), and fed chopped herring muscle to satiation every 2–3 days for 3 wk. Herring muscle contains ~30–50 and 3.0 μmol/g of TMAO and TMA, respectively (reviewed in Ref. 11). These animals consumed ~4–6% of their initial body weight per feeding, giving a nominal ration of 2% of body wt/day.
After a 3-wk acclimation period, food was withheld for 6 days, with fish being weighed while immersed in seawater on the 5th day of fasting. After the fasting period, fish were fed a single meal of chopped herring muscle and the total amount of food consumed by each animal was recorded (~2% of body weight). One hour postfeeding, fish were transferred to individual aquaria, as described above, and after approximately 1 h, an initial seawater sample was taken and frozen (~20°C). Seawater samples were also taken at 18 h posttransfer and frozen. The difference in the concentration of TMAO, urea, and ammonia between this point and the initial seawater sample was used to calculate whole animal efflux (this was day 1 of the experiment). After being in the aquaria for 24 h, the fish were removed, the aquaria were emptied and rinsed thoroughly, first with freshwater then with filtered seawater. Animals were weighed in seawater and returned to their individual aquaria, which were filled with nine volumes of aerated filtered seawater, and the above seawater sampling protocol was repeated. This procedure was repeated for a total of six experimental days, and seawater osmolality did not vary (~920 mosmol kg H2O) over the duration of the experiment.

Turnover of [14C]TMAO in free-swimming skates. The turnover and loss of [14C]TMAO in free-swimming skates were determined by a modification of the method used by Goldstein and Palatt (10). Skates were randomly selected from the main population and moved to 1 m² flow-through seawater tanks (7–9°C) with 3–6 fish per tank. Fish were fasted for 5–6 days and then anesthetized with eugenol (0.011%) in seawater as already described. After being weighed, each fish was injected intraperitoneally with a solution of [14C]TMAO (2 μCi/ml) in 50 mM HEPES (pH 6.5) to a dose of 10 μCi/kg and then returned to the 1 m² tank.

Fish were sampled at 1, 2, 7, 14, and 21 days postinjection. After being anesthetized with eugenol (0.01%), specimens were weighed and then killed by pithing. Blood was drawn into heparinized syringes and loss of [14C]TMAO in free-swimming skates were determined by the in vitro oxygenation of [14C]TMAO to TMAO with the liver microsome fraction using a modification of the radioisotopic assay previously reported (25). The modifications included as potential activators tyramine (5 mM) and octylamine (1 mM), which, like methimazole, acts as a general FMO substrate oxidizing TMAO in the toluene phase. No significant radioactivity could be found in the toluene, confirming negligible TMAO contamination. However, using the reduction mixture used in the conversion of TMAO to TMAO for the spectrophotometric assay, outlined below, followed by the addition of KOH and extraction with toluene, resulted in >90% recovery of the radioisotopic assay in the toluene phase.

In vivo oxygenation of [14C]TMAO seawater. Samples (0.5 to 1.0 ml) were mixed with 10 ml of scintillation fluid (Ecolume) and assayed for total radioactivity by scintillation counting. Samples with significant radioactivity, at 18 and 24 h posttransfer, were further analyzed for the presence of nonvolatile radioactivity, which is indicative of the likely presence of [14C]TMAO. Seawater samples were alkalized by the mixing the sample with 2 ml of 0.1 M NaOH followed by evaporation to dryness as described below for the in vitro oxygenation of TMAO.

For the analysis of tissues, samples were homogenized in 9 or 19 volumes of ice-cold 5% (wt/vol) TCA and left on ice for 10 min to precipitate proteins. Homogenates were centrifuged at 15,600 g at room temperature for 5 min, and the supernatant was collected for analysis of radiolabeled TMAO and TMAO. Total and nonvolatile [14C]-labeled radioactivity was determined for tissue TCA extracts following the same methodology used for seawater samples.

Enzyme assays. Flavin-containing monoxygenase (FMO, EC 1.4.13.8) was assayed in fractions from liver using a modification of the assay developed by Dixit and Roche (5), similar to that used by Schlenk and Li-Schlenk (19). The assay medium was based on the assay for TMAO oxidation previously (25) and included 100 mM Tris (pH 8.5 at 25°C), 50 mM KCl, 1 mM EDTA, 0.2 mM NADPH, 0.06 mM DTNB, 0.025 mM DTT, and 10 mM methimazole (omitted for control rates). The FMO activity at 25°C was determined as the rate of methimazole sulfoxidation and was calculated by subtracting the change in absorbance at 412 nm in control assays (no methimazole) from those run in parallel containing 10 mM methimazole using an extinction coefficient of 28.2 (5). Lipophilic primary amines, such as octylamine and tyramine, are known to activate cod liver TMAO oxygenation (1), as well as some mammalian FMOs (34). We included as potential activators tyramine (5 mM) and octylamine (1 mM), which when included were preincubated for 5 min prior to monitoring the rate of absorbance change. These concentrations were found to give maximal activation.

Schlenk and Li-Schlenk (19) report that an equimolar concentration of TMA strongly inhibits FMO activity, measured as methimazole oxidation, in microsomes from spiny dogfish. We also tested for inhibition of FMO activity using TMA, which may suggest the capacity for TMAO to act as an FMO substrate in skate. Inhibition by thiourea, which, like methimazole, acts as a general FMO substrate because it can be oxidized by a wide range of FMO isoforms, was also assessed. Of note, the methimazole FMO assay is particularly useful for such studies because of all substrates and potential substrates present in the assay, only the product of methimazole sulfoxidation reacts to result in a change of absorbance at 412 nm in the presence of reduced DTNB. For inhibition studies, microsomes were incubated at 25°C in methimazole free assay medium along with inhibitor (10 mM) for 15 min before assaying for FMO activity.

The in vitro capacity for TMAO oxygenation to TMAO was investigated at 25°C with the liver microsome fraction using a modification of the radioisotopic assay previously reported (25). The modifications include using 0.2 mM NADPH rather than 0.3 mM and the activity of [14C]TMA in the assay was increased from 0.1 μCi/ml to 0.25 μCi/ml. The effects of known activators of cod liver TMAO oxygenation (1), tyramine (5 mM), and octylamine (1 mM), were also assessed using microsomes.

Plasma osmoregulatory parameters. Osmolality was determined using a vapor pressure osmometer. For determining urea and TMAO,
plasma samples were deproteinized by adding nine volumes of ice-cold 5% (wt/vol) TCA followed by centrifuging for 5 min at 15,600 g. The supernatant was collected and assayed immediately or frozen (−20°C) for later analysis. Freezing had no effect on values. The TCA extract was appropriately diluted further with 5% TCA and assayed by the spectrophotometric assays of Rahmatullah and Boyde (16) and a modification of the method of Wekell and Barnett (26), as described previously for use with small sample volume (21) for urea and TMAO, respectively. Of note, TMA is typically low in elasmobranch tissues compared with TMAO (24, 28), and as such, values were not corrected for endogenous TMA.

**Seawater analysis.** Frozen seawater samples were thawed on ice and assayed directly for TMAO, urea, and ammonia. For TMAO, the higher-resolution modification of the spectrophotometric assay of Wekell and Barnett (26) described in detail by Treberg et al. (25) was used. Urea and ammonia were assayed using the spectrophotometric assays found in Refs. 16 and 21, respectively.

**Tissue TMAO, urea, and water content and [14C]TMAO specific activity.** Plasma TMAO and urea concentrations were determined as described above. Plasma was measured directly for radioactivity by mixing with 10 ml of scintillation fluid (Ecolume) followed by scintillation counting. In all cases, scintillation data were converted from counts per minute to disintegrations per minute (dpm), with the quench correction based on the counting efficiency of external standards. Tissue water content was determined by drying samples to a constant mass at 70°C under vacuum. For TMAO and urea contents, tissues were homogenized in 9 or 19 volumes of ice-cold TCA followed by centrifuging for 5 min at 15,600 g. The supernatant was collected and frozen (−20°C) for later analysis as described above for plasma TMAO and urea concentration. Freezing had no effect on values, and TMAO content was not corrected for endogenous TMA. Aliquots of the TCA extract were mixed with 10 ml of Ecolume, and the radioactivity due to the presence of [14C]TMAO was assessed by scintillation counting, allowing for the specific radioactivity of TMAO (in dpm/μmol) to be determined for each sample.

**Statistical analysis.** When appropriate, data were analyzed using an ANOVA for repeated measures (serial plasma sampling and excretion experiments). For comparisons of means, significance was assessed by one-way ANOVA or paired t-test, using Tukey’s honestly significant difference post hoc adjustment for multiple comparisons, with \( P \leq 0.05 \) being considered significant. When necessary, data were log-transformed to meet the requirement of homoscedasticity. Calculating the plasma [14C]TMAO loss coefficient was done by nonlinear regression, with \( P \leq 0.05 \) being considered a significant relationship. Values are presented as means ± SE (number of individuals).

**RESULTS**

**Effects of Food Deprivation on Plasma TMAO, Urea, and Osmolality**

When held without food for 45 days, skates maintain their plasma hyperosmotic to the seawater (920–925 mosmol) over the duration of the experiment (Fig. 1). Thus skates displayed no apparent disruption of osmoregulatory capacity. Similarly, plasma urea and TMAO concentrations were unaffected by food deprivation (Fig. 1).

**Investigations on endogenous synthesis of TMAO.** Skates injected intraperitoneally with [14C]TMAO begin showing significant whole body clearance of the radiolabel TMAO after 12 h (Fig. 2A). Clearance continued at a relatively consistent rate with ~15% of the total initial dose excreted 24 h postinjection. None of the excreted radioactivity was recoverable in the nonvolatile fraction, indicating that no labeled TMAO was excreted. All tissues examined have notable radioactive content (Fig. 2B); however, no significant radioactivity was recoverable in the nonvolatile fraction, indicating a lack of detectable endogenous TMAO oxygenation. Because of these results, it was concluded that no detectable TMAO synthesis was occurring in vivo, and in vitro studies were conducted because they may provide a higher level of resolution.

The crude microsomal fraction had detectable FMO activity, as measured by methimazole sulfoxidation, and there was significant activation of FMO with the lipophilic primary

![Fig. 1. Plasma osmotic parameters in winter skates held without food. No differences were found over time. Values are means ± SE (n = 8). TMAO, trimethylamine oxide.](http://ajpregu.physiology.org/content/291/DECEMBER/2006/R1793/F1)

![Fig. 2. A: whole body clearance of [14C]trimethylamine (TMAO) following intraperitoneal injection (dose of 50 μCi/kg) in winter skates. B: tissue radioactivity due to the presence of [14C]TMAO, 24 h postinjection. Dotted line indicates expected radioactivity assuming complete equilibration of label (i.e., 0.05 μCi/g). No radioactivity was found to be attributable to the presence of [14C]TMAO in the seawater (A) or tissue analysis (B). Values are means ± SE (n = 3).](http://ajpregu.physiology.org/content/291/DECEMBER/2006/R1793/F2)
amines octylamine and tyramine (Fig. 3A). Although the microsomal fraction used in this study was not obtained via the traditional 90,000 to >100,000 g centrifugal force, using tyramine as an activator, we demonstrated a more than fivefold enrichment of FMO activity compared with the initial 20,000 g supernatant (Fig. 3B). In the presence of tyramine, methimazole sulfoxidation was not inhibited by an equimolar concentration of TMA but was significantly inhibited by thiourea, the latter of which is another broad FMO substrate (Fig. 3C). Similar results were found with octylamine; however, we found activity was too low in the absence of activators [0.18 ± 0.03 nmol·min⁻¹·mg protein⁻¹ (n = 4)] to reliably quantify inhibition kinetics.

Using the crude microsomal fraction, we attempted to measure TMA oxygenation but were unable to detect significant activity even in the presence of the FMO activators octylamine (1 mM) or tyramine (5 mM); the lower limit of detection was ~0.02 nmol·min⁻¹·mg protein⁻¹. That is, the lower limit of detection for TMA oxygenation is ~10-fold lower than measured activity for the spectrophotometric FMO assay with methimazole as substrate. Thus, consistent with the in vivo study, it was concluded that, despite detectable FMO activity, TMA oxygenation is an unlikely source of TMAO in winter skates.

**Whole animal excretion experiments.** Exploratory experiments demonstrated that whole animal TMAO excretion was measurable 48 h after the last feeding for skates fed to saturation every 2–3 days [13 ± 3.8 μmol·kg⁻¹·h⁻¹ (n = 3)]. Of note, the assay used measures of TMA, after the reduction of TMAO to TMA, followed by extraction into toluene. Because the low rates of TMAO excretion found are already on the lower end of sensitivity of the assay, we did not attempt to distinguish how much of the measured efflux was due to TMA. As such, although we refer to TMAO efflux for simplicity, it is important for the reader to appreciate the possibility that TMA may make up some proportion of this measurement, especially given the notable excretion of radiolabeled TMA in the previous experiment (see Fig. 2). When winter skates were fasted for 6 days, seawater samples taken ~1 h posttransfer displayed an initial measurable increase in absorbance at 412 nm (the absorbance used to measure the TMA-picric acid complex formed) compared with blanks run with filtered seawater. This indicates the presence of TMA, TMAO, or some unknown false positive, which is toluene soluble and reacts with picric acid to increase the absorbance at 412 nm. However, and essential for this study, there was no detectable increase in absorbance at 412 nm with the toluene extract up to 24 h posttransfer. This indicates that the TMAO (plus TMA) concentration in the seawater did not measurably increase over time in fasted winter skates [lower limit of detection ~2 μmol·kg⁻¹·h⁻¹; data not shown, (n = 3)]. Neither urea nor ammonia displayed this initial bolus efflux response, making it unlikely that this was due to either a transient increase in excretion or epithelial permeability (data not shown).

Fish were fasted for 6 days, followed by being fed a single meal (~2% of body weight) of herring muscle to determine the effects of a single feeding event on TMAO excretion. These fish initially displayed detectable TMAO excretion levels for 3 days after feeding, but this was reduced to below the limits of detection for four animals on day 4 of the experiment and by day 5, TMAO excretion was undetectable in all but one skate (Fig. 4). The rate of urea efflux was quite variable among animals and did not significantly differ following a single feeding event (Fig. 4), although the average value on day 3 was almost twofold higher than on day 1. Urea excretion had a trend of increasing over the first 2 days postfeeding and was significantly elevated on day 3, with rates sharply decreasing on day 4 to the initial level (day 1). Feeding had no effect on the %ureotelism (% of total nitrogen efflux via urea and ammonia, that is due to urea—data not shown, and relative to the total nitrogen losses due to urea efflux and ammonia excretion TMAO does not appear to play a significant role in nitrogen excretion.

**Turnover of [¹⁴C]TMAO in free-swimming skates.** The observed rate of directly measured TMAO efflux in skates maintained in small aquaria—and thus with restricted capacity for movement—was much lower than would be predicted based on the results of Goldstein and Palatt (10). Thus we reassessed whole animal TMAO efflux using methodology similar to that used by Goldstein and Palatt (10). This involves injecting and
subsequently monitoring the loss of injected [14C]TMAO to estimate the rate of whole animal TMAO loss.

The decline in the specific activity (dpm/μmol) of plasma TMAO followed a biphasic pattern, with both components intercepting at 7 days postinjection (Fig. 5A). The calculated loss coefficients (day⁻¹, which approximates the fraction of the entire labeled TMAO pool lost per day) were 0.064 and 0.012 for the time periods from days 1–7 and days 7–21, respectively. However, the tissue TMAO pools continued to mix with the extracellular pool, as indicated by lower tissue TMAO-specific activities than in the plasma (Fig. 5B). This slow equilibration, requiring around 7 days in the case of liver, kidney, and intestine and much greater than 21 days for muscle, results in an overestimation of the loss coefficient because a component of the observed decline in the labeled plasma TMAO is due to uptake into the tissues rather than loss to the environment.

**Tissue TMAO levels and exchange: relative permeability of tissues to TMAO.** There were no significant trends in plasma or tissue TMAO concentrations over the duration of the loss-coefficient experiment (Fig. 5C). With the concentration of TMAO remaining relatively stable in the plasma and tissues over the experiment, it can be assumed that for every molecule of TMAO exchanged from the radiolabeled extracellular fluid (ECF) pool into the intracellular fluid (ICF), there is a coincident exchange of a molecule of TMAO from the ICF pool to the ECF. Otherwise, the tissue TMAO levels would have to be variable over time. The time required for the specific activity of TMAO in a tissue to equal that in the plasma gives an indication of the rate of exchange between the total tissue TMAO pool (ICF plus ECF) and the pool of ECF TMAO, represented by the plasma (Fig. 5B). The time required for equilibration can also be considered a relative permeability index of the cell membranes in a tissue to TMAO. Given the above rationale, the tissues can be ranked based on their relative permeability to TMAO as follows: kidney ≈ liver ≫ spiral valve ≫ muscle (Fig. 5B).

**Tissue TMAO and urea levels: comparison with plasma concentration.** The concentration of urea in the plasma of skates used in the [14C]TMAO turnover experiment did not show a trend of decreasing with time when held without food for between 7 and 27 days (data not shown). This is consistent with the initial food-deprivation experiment (Fig. 1). The urea concentration in the tissues (μmol/ml of tissue water) also did not change over the experiment (data not shown), and data from all sample points were pooled. The ratio of tissue to plasma urea

![Fig. 4. Whole animal eflux of urea, ammonia, and TMAO (as described in RESULTS, TMAO, and TMA combined) for winter skates after a single ration (2% of body weight) of herring muscle on day 1 of the experiment. Values are means ± SE (n = 8). Of note, TMAO eflux was detectable in all animals for days 1–3; detectable in four animals on day 4 and in one animal for days 5–6. Undetectable rates were entered as 0 for graphical purposes only. *Significant difference from day 1 (P ≤ 0.05). †Trend toward differing from day 1 (P ≤ 0.1).](image-url)

![Fig. 5. A: plasma-specific activity (dpm/μmol) of TMAO after intraperitoneal injection (dose of 10 μCi/kg) of [14C]TMAO. The loss coefficient (unit of day⁻¹) for days 1–7 and 7–21 is 0.064 and 0.012, respectively. B: tissue specific activity (dpm/μmol) of TMAO relative to plasma value (unity between values indicated by horizontal line). *Significantly lower (P ≤ 0.05) than plasma-specific radioactivity. C: TMAO concentration of plasma and tissues (μmol/ml of plasma or tissue water). n = 4, 3, 4, 5, 5 for days 1, 2, 7, 14, and 21 postinjection, respectively.](image-url)
shows that muscle, liver, kidney, and spiral valve have similar concentrations of urea in the ICF and ECF. Although the ratio is close to unity between the ECF and ICF, it is significantly higher than 1 for the latter three tissues (Fig. 6) with liver having the highest urea concentration. The tissue-to-plasma TMAO ratio is >1 for all tissues examined, indicating that TMAO is accumulated intracellularly. The tissue-to-plasma TMAO ratio was higher than the same ratio for urea in all tissues.

**DISCUSSION**

**Maintaining TMAO levels in skates: the importance of retention over synthesis.** The primary finding of this study is that winter skates can maintain high levels of TMAO in the absence of an exogenous input. We found no evidence supporting endogenous synthesis of TMAO, and the maintenance of TMAO appears to be due to very low whole animal losses, and thus turnover, of this solute. These data warrant specific discussion because they conflict with the conclusions of both Schlenk (18) and Goldstein and Palatt (10).

The presence of FMO activity does not confirm TMA oxygenation. We found FMO activity in skate liver microsomes, 0.18 nmol·min⁻¹·mg protein⁻¹ (measured as methimazole oxidation) in the absence of activators. This value compared reasonably well with data on dogfish microsomal FMO activity (0.63 nmol·min⁻¹·mg protein⁻¹), also measured as methimazole oxidation (19). However, we come to profoundly different conclusions on significance of this FMO activity.

In a review on the occurrence of FMO in nonmammalian eukaryotes, Schlenk (18) suggests FMO activity and FMO-like proteins, which are recognized by antibodies for mammalian FMO1 and FMO2, likely play a role in the synthesis of TMAO for osmoregulation in fishes. Indeed, this is also the role Schlenk and Li-Schlenk (19) attributed to elasmobranch liver FMO activity, largely based on inhibition of FMO activity by TMAO. Schlenk (18) further concludes that previous studies indicating a lack of detectable TMA oxygenation may be erroneous because of the use of less sensitive assays that use crude homogenates as the enzyme source rather than an FMO-enriched microsomal fraction.

To reiterate, our data are consistent with Schlenk and Li-Schlenk (19), with regard to the presence of FMO activity in an elasmobranch that lacks apparent TMA oxygenation. However, we found no evidence, either direct, indirect, in vivo, or in vitro, for endogenous TMAO synthesis via TMA oxygenation. This includes no inhibition of liver microsomal FMO activity by an equimolar concentration of TMA (Fig. 3). We did find significant inhibition of methimazole oxidation by equimolar thiourea, which is another broad FMO substrate, again supporting the presence of FMO or at least an FMO-like enzyme in winter skate liver.

Although it is clear that inhibition was noted by Schlenk and Li-Schlenk (19), the nature of the TMA inhibition of FMO (19) is also in our view inconclusive. The “competitive” inhibition of dimethylaniline oxidation by TMA appears to have been assessed by double-reciprocal plots and is largely dependent on inclusion of data at the lowest substrate concentration (see Fig. 2 in Ref. 19). These data are notable for having the highest error associated with transformation for kinetic analysis using a double-reciprocal plot. Furthermore, there were no data under control assay conditions (i.e., no inhibitor) at the same low substrate concentration and thus no confirmation that the double-reciprocal transformation is suitable for kinetic analysis at low-substrate concentration. With respect to our competition studies, we found inclusion of tyramine in the assay was required to reliably reproduce FMO activity that was high enough to perform inhibition studies. Although it is possible that tyramine could in some unknown way interfere or mask TMA inhibition of FMO, it is unclear why this would not also occur with thiourea, which did inhibit methimazole oxidation.

In defense of past studies using crude homogenates, rather than microsomes, for radioisotopic assays of the conversion of TMA to TMAO, we found the lower limit of detection for TMA oxygenation (0.02 nmol·min⁻¹·mg protein⁻¹) to be ~10 and >100-fold lower than measured FMO activity in microsomes in the absence of activators or in the presence of tyramine, respectively. Because of spectral interference at high microsomal protein concentration with the FMO assay, we found the radioisotopic assay for TMA oxygenation is ~5- to 10-fold more sensitive than the spectrophotometric assay on a milligram per protein basis (data not shown). Thus we feel the radioisotopic assay has the required sensitivity even with crude homogenates to detect significant formation of TMAO.

**The relationship between FMO activity and TMA oxygenation in elasmobranchs.** Taken as a whole, the above argues against a global capacity for significant endogenous TMA oxygenation in elasmobranchs. Although we agree that the in vitro oxygenation of TMA is most likely catalyzed by an FMO, or at least an FMO-like enzyme, in elasmobranchs, it is our contention that the presence of FMO activity, when measured only with broad FMO substrates, such as methimazole and dimethylaniline, should not be taken as an indicator of TMA oxygenation in elasmobranchs. Instead, we propose an alternative hypothesis, in which all elasmobranchs possess FMO activity but only some have the isoform(s) that TMA acts as a physiological substrate. This hypothesis is an extension of the recently proposed phylogenetic explanation for the distribution of TMA oxygenation in elasmobranchs (25) and is based on 1) all elasmobranchs examined to date have displayed FMO activity when substrates with broad isoform reactivity are used and 2) many species with FMO activity lack significant TMA oxygenation (2, 7, 9, 25, and present study). While the above discussion fits the currently available data, at present, there is
a need for more information by extending the numbers of species studied, as well as, including members from orders and families not yet represented. Furthermore, additional molecular and functional characterization of purified, or partially purified, elasmobranch FMO(s) is also needed to properly delineate the isoforms present, along with their physiological properties.

**Whole animal TMAO losses.** Goldstein and Palatt (10) utilized the clearance of $[^{14}C]$TMAO from the plasma to measure the whole animal loss of TMAO in several marine elasmobranchs. This methodology is based on the assumption that there is a rapidly equilibrating pool of TMAO in the body, and following the injection of a tracer, this pool quickly mixes. Once mixed, the rate of loss of the labeled tracer is representative of the proportion of whole body TMAO clearance that can be attributed to the rapid mixing pool if, over the duration of the experiment, the levels of TMAO and the relative enrichment of all body TMAO pools does not change (i.e., animals are in a steady state).

Goldstein and Palatt (10) injected elasmobranchs intravenously with labeled TMAO and monitored the decline in plasma radioactivity due to $[^{14}C]$TMAO over a period of 6 days. For spiny dogfish and little skate, species lacking the capacity for TMA oxygenation, it was concluded that the model was appropriate and the loss coefficient was determined to be 0.10 and 0.078 day$^{-1}$, respectively (10). The present study on winter skates found a loss coefficient for $[^{14}C]$TMAO in the plasma of 0.064 day$^{-1}$ over the first 7-day period of the experiment, a value remarkably similar to that reported by Goldstein and Palatt (10) for the congeneric little skate. However, from 7 to 21 days postinjection, the loss coefficient drops to 0.012 day$^{-1}$, in other words, ~1% of total body TMAO lost per day. While TMAO levels were stable over the 21-day experiment, the intracellular TMAO (TMAO$_{ICF}$) pools continue to mix with extracellular TMAO pool (TMAO$_{ECF}$) over the experiment (Fig. 5B). The substantially higher TMAO loss coefficient from days 1 to 7 is likely a significant overestimate of whole animal TMAO loss due to the continued mixing of TMAO$_{ECF}$ and TMAO$_{ICF}$ pools, in most tissues, including liver, intestine, and kidney. A marked decline in the calculated TMAO loss coefficient followed this initial mixing period (Fig. 5A). The muscle TMAO$_{ICF}$ pool continued to mix with the TMAO$_{ECF}$ over the duration of the experiment, reaching ~40% the specific activity of the TMAO$_{ECF}$ pool. Muscle is the largest tissue in skates (~40% of total mass), and the continued mixing of muscle TMAO$_{ICF}$ and the extracellular TMAO pool indicates that the loss of 1% of total body TMAO is still an overestimate, with some of the apparent loss being due to dilution of the labeled TMAO$_{ECF}$ pool with unlabeled TMAO$_{ICF}$ from the muscle.

In a study of TMAO turnover in the teleost Gadus morhua, Ágústsson and Ström (1) found a whole animal loss for $[^{14}C]$TMAO of 0.5% day$^{-1}$ following an initial rapid loss for about 7 days postinjection of the label. These authors also attributed this initial rapid loss phase to the mixing and compartmentalization of TMAO to the intracellular compartment (1). Indeed, Ágústsson and Ström (1) questioned the very high loss coefficients reported for elasmobranchs (10) because the duration of the experiment used to determine losses appeared to be too short to accommodate for the slow equilibration of TMAO in fishes.

**TMAO and nitrogen excretion in elasmobranchs.** On the basis of the results of Goldstein and Palatt (10), it has been suggested that TMAO may have a significant contribution to total nitrogen excretion in elasmobranchs (29). Our results argue that TMAO and/or TMA losses are a negligible contribution toward nitrogen excretion. In fed skates, the measured rate of whole animal TMAO (plus TMA) efflux is only ~1% that of the nitrogen losses due to the combined contributions of urea and ammonia (Fig. 4). This contribution is even less in fasted skates where TMAO efflux is below the limits of detection using the methodology of the present study. Although not the primary goal of this study, our results shed some light on two other concepts that have been proposed in regard to nitrogen balance in elasmobranchs. It has been suggested that because of high urea losses at the gills, marine elasmobranchs may be so nitrogen limited [reviewed by Wood (29)] that they would display little or no postprandial increase in nitrogen excretion. Mømmsen and Walsh (14) have also hypothesized that any excess exogenous nitrogen (i.e., dietary) would be excreted as ammonia, rather than urea, which would have to be first synthesized at the cost of ATP prior to excretion. If we assume ~1,600 μmol-N per gram in the teleost muscle supplied as food (30) and a ration of 2% of body weight, the increase in ammonia excretion would account for no more than about 8% of the total ingested nitrogen, an increase of approximately [40 μmol ammonia-N·kg$^{-1}$·h$^{-1}$·× 24 h (days 1 and 2)] + [70 μmol ammonia-N·kg$^{-1}$·h$^{-1}$·× 24 h (days 2 and 3)] = 2,640 μmol-N/kg excreted above basal levels compared with [(1,600 μmol-N/g of food) × (1,000 g of skate × 2% ration)] = 32,000 μmol-N ingested/kg. Our results support both of the above theories and are also consistent with a recent feeding study on spiny dogfish that found that there was no postprandial increase in urea efflux along with a slight increase in ammonia excretion following a ration of 2% minced teleost muscle (30).

**Tissue uptake and exchange of TMAO: evidence for active uptake.** By comparing the concentration of a compound in a tissue (combined ECF and ICF) relative to the plasma concentration (ECF only), it is possible to determine whether the compound is preferentially excluded or accumulated in the ICF. For example, there is little or no concentration gradient for urea between the ECF and ICF in tissues from the winter skate (Fig. 6). Conversely, all tissues examined had substantial TMAO accumulation within the ICF (Fig. 6).

Because the level of TMAO in tissues did not vary over the experiment (Fig. 5), we can assume that the rate at which the TMAO$_{ECF}$ and TMAO$_{ICF}$ pools equilibrate is a relative indication of the leakiness of a tissue’s plasma membrane to TMAO. Note that the apparently lower degree of TMAO accumulation in the kidney ICF is likely due to the proportionally higher ECF volume of fish kidneys compared with other tissues. Measureable exchange between the TMAO$_{ECF}$ and TMAO$_{ICF}$ pools along with the TMAO$_{ICF}$ being at a significantly higher concentration indicates there is evidence for an active TMAO uptake mechanism that occurs in all tissues examined in this study. An active TMAO transporter has been described in bacteria (17); however, to our knowledge, TMAO transport in elasmobranchs has only been studied in the red blood cell, and efflux appears to be facilitated by the volume-activated organic osmolyte channel (13). The organic osmolyte channel of the elasmobranch red blood cell allows for facili-
tated diffusion, not active transport, and as expected, the concentration of TMAO is roughly equal between the plasma and the ICF of the red blood cells of winter skates. As such, there appears to be a novel transport mechanism in the tissues of elasmobranchs in which a substantial TMAO gradient is maintained between the ECF and ICF.

In conclusion, winter skates can maintain TMAO levels for several weeks without an exogenous supply. While the liver of winter skates demonstrated enzymatic capacity for methimazole sulfoxidation, indicative of FMO activity, no evidence for TMAO oxygenation was found. Whole animal TMAO losses are very low, confirmed by direct efflux measurements and [14C]-TMAO loss coefficient in fed and unfed skates, respectively. Daily losses are likely <1% of whole body TMAO per day in fasting winter skates. As such, we conclude that winter skates lack endogenous TMAO synthesis and rely on low whole animal losses to maintain TMAO levels between feeding bouts.

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