Trimethylamine oxide excretion rates in elasmobranchs

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METHODS

Animals and procedures. Spiny dogfish and little skates were caught by handline and otter trawl, respectively, off Mount Desert Island, Maine. Nurse sharks and lemon sharks were captured by handline in the vicinity of Bimini, The Bahamas. Experiments on the first two species were conducted at the Mount Desert Island Biological Laboratory, where the fish were kept in aquaria maintained at 12.14°C. Investigations on the latter two species were carried out at the Lerner Marine Laboratory, where the fish were maintained in aquaria at approximately 27°C.

Fish were injected intravenously with approximately 5 μCi [14C]TMAO/kg (sp act, 0.83–1.65 μCi/μmol), which was synthesized from [14C]trimethylamine (New England Nuclear) according to the procedure of Hickinbottom (9). The purity of the synthesized [14C]TMAO was checked by converting it to [14C]TMA with TiCl4 (7), isolating the radioactive base, and assaying its radioactivity by liquid scintillation spectrophotometry. More than 90% of the radioactivity in the synthesized product was [14C]TMAO.

Blood samples were drawn from a caudal vessel 4–24 h after injection and at 24-h intervals for 6 days. On the 3rd day of the experiment the fish (except skates) were anesthetized with hexobarbital sodium (Sigma Chemical Co., 15 mg/kg, iv) and placed on an operating table; their gills were continuously perfused with flowing seawater. A muscle biopsy (approximately 0.2 g) was taken through a dorsal midline incision (about 3 cm long). The wound was filled with Gelfoam (Upjohn) and sutured. The entire procedure took about 15 min. The fish maintained spontaneous branchial movements during the operation and recovered from anesthesia (as judged by swimming movements) within 5–10 min after they were returned to the aquaria. Muscle biopsies (as well as other tissue samples in the dogfish) were taken after anesthesia of the fish on the last day of the experiment (day 6). A limited number of experiments were conducted on nurse sharks in which blood samples and muscle biopsies were taken at early time points—days 1, 2, and 3 after injection of [14C]TMAO.

Radioactive and chemical assays. Blood samples were centrifuged in a clinical centrifuge and the plasmas removed. Plasma protein was precipitated with cold trichloroacetic acid (final concentration, 5%). After centrifugation, the supernatant fluid was extracted with ether to remove trichloroacetic acid and analyzed for chemical TMAO and [14C]TMAO as described previously (6). Tissue samples were analyzed for [14C]TMAO by dissolving 100 mg tissue...
in 1 ml of tissue solubilizer (Protosol, New England Nuclear), suspending the mixture in 15 ml of either Aquasol (New England Nuclear) or a toluene based cocktail (Omnifluor, New England Nuclear), and counting the mixture in either a Packard Tricarb or Nuclear Chicago Mark I liquid scintillation counter. Samples were corrected for quenching with the aid of an external standard. After tissue samples had been dissolved in concentrated KOH and then neutralized with perchloric acid, chemical TMAO was assayed by microdiffusion titration (7).

**Determination of [14C]TMAO loss coefficient.** Based on certain simplifying assumptions, a mathematical model applicable to all species studied was developed to determine rates of loss of TMAO from an equilibrated internal compartment (see Appendix A). The equations of the model were solved to give:

\[
C_t = C_0 \exp \left( \frac{-Kt}{V_2} \right)
\]

where \(C_t\) is concentration of [14C]TMAO (dpm/ml) in the internal compartment at time \(t\) (days), \(C_0\) is the concentration of [14C]TMAO in the compartment at \(t = 0\), \(K\) is a permeability coefficient (ml/day), and \(V_2\) is the volume of the internal compartment in which TMAO is distributed (ml). Losses are expressed in terms of a loss coefficient \((-K/V_2)\) having units of days\(^{-1}\).

**TMAO excretion in skates.** Excretion of TMAO was measured directly in skates after the final blood samples were taken on day 6 of the experiment. Skates (0.7-1.0 kg) were placed in plastic aquaria containing 3 liters of aerated seawater and maintained at 12-14°C for 2 h. Water and plasma (derived from blood taken from a caudal vessel) samples were analyzed for radioactive TMAO. Plasma samples were also analyzed for chemical TMAO. These values were used to calculate total excretion rates of TMAO using the equation:

\[
\text{µmol TMAO excreted/kg} \cdot \text{min} = \frac{\text{dpm [14C]TMAO excreted/kg} \cdot \text{min}}{\text{dpm [14C]TMAO/mL plasma}} \frac{\text{µmol TMAO/mL plasma}}{
\]

RESULTS

Figure 1 shows the pattern of loss of [14C]TMAO from the plasma spaces of four elasmobranch fishes after intravenous injection of the radioisotope. Subsequent to the 1st day after injection, the rates of loss of [14C]TMAO follow first-order kinetics; there is a logarithmic decrease in [14C]TMAO concentration with time. These data suggest that after a 24-h equilibration period the loss of [14C]TMAO from the plasma is due to exit of the compound from a single internal compartment to the environment and that filling of the internal compartment had reached a steady state by day 1. Support for this assumption was obtained by demonstrating that the concentration of [14C]TMAO in muscle (which comprises approximately 40% of total body wt (3)) relative to that in plasma remained constant between day 3 and day 6 of the experiment in all fish, with the possible exception of the lemon shark (Table 1). The lemon shark data are discussed in greater detail below. Furthermore, in a separate experiment (Table 1, exp. A) we found that the relative concentration of [14C]TMAO in the muscle of the nurse shark reached a steady-state level by 1 day after injection of the radioisotope. Although these experiments demonstrated that the relative concentration of [14C]TMAO in muscle reached a steady state soon after injection of the isotope, it was found that the isotope did not completely equilibrate with the TMAO pools in muscle even after 6 days. The specific activity of [14C]TMAO in muscle ranged from 40% to 60% of the plasma specific activity in the four elasmobranchs examined (Table 1). In a separate experiment done in the dogfish we found that 6 days after injection of [14C]-TMAO the relative specific activity of [14C]TMAO in tissue was similar to that in plasma, for the skin and kidneys, but not for muscle, cartilage, and liver (Table 2). The lack of equilibration of [14C]TMAO in muscle and other tissues in the presence of steady-state levels of the isotope indicates that there are one or more pools of slowly exchanging chemical TMAO in these tissues that equilibrate with plasma TMAO over periods of time that are long compared to the time of the experiment (6 days).

Loss coefficients of [14C]TMAO for the four elasmobranchs examined in this study are shown in Table 3. The magnitude of these coefficients varies from 0.044 in the nurse shark to 0.138 in the lemon shark. The loss coefficients in the skate and dogfish are intermediate, being...
from the plasma of the lemon shark went to an unequili-
creted at a more rapid rate and biosynthesis of the compound was necessary to maintain the elevated levels of the osmoregulatory agent in the plasma. However, in experiments designed to test this theory we found no relation between rates of excretion and the ability to biosynthesize TMAO in the four elasmobranchs examined. In the dogfish and little skate we estimated the rate of loss of TMAO from the body fluids to be 7.8 and 10.4%/day, whereas in the lemon shark and nurse shark, two elasmobranchs that are able to biosynthesize TMAO, the loss rates were 13.8 (or a corrected value of 7.0%) and 4.4% (Table 3).

The method used to determine the rate of excretion of TMAO was an indirect, radioisotopic-tracer procedure. We decided to use this procedure in order to avoid the deleterious effects of restraining the lemon sharks and dogfish, both of which must keep swimming for proper ventilation. In this method the rate of loss of [14C]TMAO from the extracellular fluid, after an initial period of distribution of the compound in the body fluid compartments, is taken to reflect the rate of excretion of TMAO from the fish. In a previous study, using the indirect, radioisotopic-tracer procedure, Goldstein and Forster (5) found that the rate of loss of [14C]urea from the body fluids of the little skate was 2.0%/day. Since the concentration of total urea in the body fluids of this fish is approximately 400 mmol/kg, the rate of excretion of urea would be 330 μmol/kg × h. This value compares favorably with that determined directly in skates maintained in aquaria—240 μmol/kg × h (5), supporting the validity of the radioisotopic method for measuring rates of excretion in free-swimming fish. In the present study the rate of TMAO excretion, measured directly in skates kept in aquaria, was approximately 34 μmol/kg × h. The rate of excretion of the end product, calculated from the loss coefficient (Table 3), was approximately 84 μmol/kg × h. The higher value for TMAO (and urea) excretion rates calculated from the loss coefficients compared to that determined by the direct method may be due, as mentioned previously, to a greater rate of excretion of TMAO by free-swimming fish.

It is not unreasonable to assume that unrestrained fish would have both a more vigorous circulation and higher ventilation rates than those restrained in small aquaria and, therefore, faster rates of elimination of nitrogenous end products. Indeed we noticed that the rates of TMAO excretion fell progressively with time in the skates maintained in aquaria. On the other hand, it is difficult to rule out the possibility that the calculated loss coefficients represent something more than excretion of the compound into the environment, i.e., that there is significant loss of TMAO from the extracellular fluid to other body compartments during the 6-day experimental period. However, there are two facts that argue against the latter possibility. First, although [14C]TMAO in plasma did not equilibrate completely with the muscle compartment, which is the major compartment outside of the extracellular fluid, the labeled compound did reach a steady-state concentration in muscle during the 6-day period. The higher value of 7.0% calculated from the loss coefficients compared to that determined by the direct method may be due, as mentioned previously, to a greater rate of excretion of TMAO by free-swimming fish.

Second, the kinetics of disappearance of [14C]TMAO from plasma were first order (Fig. 1) after the 1st day. A more complex set of kinetics would be expected if there were significant filling of body fluid compartments with [14C]TMAO in addition to loss of isotope to the environment during the course of the experiment. Thus, despite the limitation of the indirect method, it is likely that the loss coefficients calculated for the four species represent a reasonable approximation of the true rate of excretion of TMAO in free-swimming fish. The purpose of this investigation was to determine whether differences exist in the rates of excretion of TMAO by elasmobranchs that can convert endogenous precursors to TMAO (the lemon and nurse sharks) and those that cannot (the dogfish and little skate). The TMAO loss coefficients calculated for the dogfish and skate lie between those calculated for the lemon shark and nurse shark. Thus, even taking into account the previously stated reservation of equating loss coefficients with rates of excretion, it is highly unlikely that there is any relation between the rate of excretion of TMAO and the ability to synthesize the compound in the elasmobranchs examined.

Thus, the differences in ability of various elasmobranchs to synthesize TMAO remain unexplained. We have considered the possibility that the presence or absence of TMAO biosynthesis in individual species may be related to dietary differences among elasmobranchs. It is possible that those species that do not synthesize TMAO endogenously may have a more plentiful supply of TMAO in the diet and do not need to synthesize the compound. However, this cannot be the only explanation, since the diets of both the spiny dogfish (Squalus acanthias) and smooth dogfish (Mustelus canis) contain an abundance of food rich in TMAO (2, 8) but only the latter species synthesizes TMAO endogenously (4, 6).

Goldstein and Funkhouser (6) originally suggested that the ability of certain elasmobranchs to synthesize TMAO may be temperature related, i.e., those fish that synthesize TMAO are found in tropical-temperate waters, and those that do not are found in the arctic-temperature zone. However, the stingray Dasyatis americana, an inhabitant of the tropical-temperate zone, does not synthesize the end product (4).

Finally, it is possible that the scattered distribution of biosynthesis of TMAO among the elasmobranchs is due to a random distribution of the “TMAO genes” in modern elasmobranch species. The ancestral form probably possessed the ability to synthesize TMAO, but the gene(s) controlling this biochemical pathway are obviously not essential to the survival of the species and may have been randomly deleted during evolution. A close analogy may be found in the evolutionary deletion of ascorbic acid biosynthesis among the mammals (1).

**APPENDIX A: MATHEMATICAL DETERMINATION OF [%]TMAO LOSS COEFFICIENT**

Any organism can be plausibly modeled as a number of interacting, macroscopic compartments. The simplest compartmental models are linear in nature. The prevalent form of solutions for linear compartmental systems is that of a sum of exponential terms (see, for instance, Jacquez, J. A. *Compartmental Analysis in Biology and Medicine*. New York: Elsevier, 1972). Thus, for linear systems, once the mathematical model is specified, the problem reduces to one of identifying exponential decay constants. Specification of the decay constants then yields information about rates of mass and/or energy exchange among the various compartments of the organism and between the organism and its environment.
If the model consists of only a few compartments (i.e., if there are only a few exponential terms in the solution to the equations of the model) and the decay constants are separated by factors of 2 or more, the technique of curve peeling is often used to obtain estimates of the decay constants from experimental data (Jacquez, p. 102ff.) This technique was used, as explained below, to obtain a value for the TMAO loss coefficient for each species we investigated.

To obtain approximate initial estimates of the rates of loss of TMAO for the different species under investigation, a simple compartmental model applicable to all the species studied (with the possible exception of the lemon shark, as discussed in the main text and in Appendix B) was constructed. The model is based on the following assumptions:

1) Each organism is composed of both fast and slowly exchanging internal compartments.

2) The fast compartments all equilibrate within 1 day (see steady-state data given in Table 1).

3) The slow compartments remain unequilibrated for as long as 6 days (see data given in Table 1).

4) Loss of TMAO from the organism to the environment is much slower than internal equilibration among the fast compartments and is entirely passive in nature.

Based on these assumptions, the simple model shown in diagram A1 was constructed and used, along with experimental data for times greater than 1 day and less than 6 days (equivalent to curve peeling) to obtain approximate values for the TMAO loss coefficients from the different species.

Diagram A1

Let $C_i$ = concentration of tracer in compartment $i$ (dpm/ml)
$V_i$ = volume of compartment $2$ (ml)
$t$ = time (days)

A simple mass balance over the system leads to

$$\frac{d(CV_i)}{dt} = -K(C_i - C_0)$$

(1)

The solution to equation 1 is

$$C_i = C_i(0) \exp \left( \frac{-Kt}{V_i} \right)$$

(2)

where $C_i(0)$ is the concentration in compartment 2 at $t = 0$ (i.e., at 1 day). Compartment 2 is taken to be all those regions rapidly equilibrating with the animal’s ECF space. The equilibration need only be rapid compared to the time course of TMAO loss from the animal and to the characteristic transport times associated with more slowly equilibrating regions (see assumptions listed above).

REFERENCES


