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Review

Trimethylamine oxide accumulation in marine animals: relationship to acylglycerol storage

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Summary

Trimethylamine oxide (TMAO) is a common and compatible osmolyte in muscle tissues of marine organisms that is often credited with counteracting protein-destabilizing forces. However, the origin and synthetic pathways of TMAO are actively debated. Here, we examine the distribution of TMAO in marine animals and report a correlation between TMAO and acylglycerol storage. We put forward the hypothesis that TMAO is derived, at least in part, from the hydrolysis of phosphatidylcholine, endogenous or dietary, for storage as diacylglycerol ethers and triacylglycerols. TMAO is synthesized from the trimethylammonium moiety of choline, thus released, and is retained as a compatible solute in concentrations reflecting the amount of lipid stored in the body. A variation on this theme is proposed for sharks.

Key words: trimethylamine oxide, choline, phosphatidylcholine, lipid, cephalopod, buoyancy, deep sea, urea, solute.

Introduction

Methylamine compounds, particularly trimethylamine oxide (TMAO), are compatible osmolytes that commonly occur in tissues of marine organisms (Yancey et al., 1982). Their concentrations vary extensively, however, among habitats and species and even with season and ontogeny within species (for a review, see Hebard et al., 1982). There are numerous hypotheses attempting to account for the distribution of methylamines. High TMAO levels in polar fishes are thought to increase osmotic concentration, thus depressing the freezing point of the body fluids (Raymond and DeVries, 1998; Raymond, 1994). Sanders and Childress (1988) and Withers et al. (1994) point out that trimethylamine (TMA) and TMAO, as a result of their large positive partial molal volumes, impart considerable lift to counteract sinking in some pelagic marine animals. TMAO is best known, however, as a ‘counteracting solute’ that protects proteins against various destabilizing forces (Yancey et al., 1982).

In elasmobranchs, for example, TMAO may counteract the toxic effects of urea on proteins (Somero, 1986; Yancey and Somero, 1980). Because they are iso-osmotic with sea water, sharks retain large quantities of urea in their fluids as an osmolyte. Urea is highly perturbing to enzyme systems. TMAO has a demonstrated ability to counteract the perturbing effects of urea on enzyme activity when accumulated in a 2:1 ratio with urea (Somero, 1986). Although some enzymes seem to have an evolved tolerance of urea regardless of TMAO (for a review, see Ballantyne, 1997), the counteracting solute hypothesis has received wide support (Wang and Bolen, 1997; Yancey et al., 1982; Yancey and Somero, 1980; Yancey and Siebenaller, 1999; Somero, 1986).

TMAO may also counteract the effects of hydrostatic pressure on enzyme function in deep-sea animals. Yancey and colleagues (Gillett et al., 1997; Kelly and Yancey, 1999) have demonstrated a correlation between TMAO concentration and capture depth in a variety of organisms. They have also shown that 250 mmol l^{-1} TMAO *in vitro* is able to counteract the loss of activity in some enzymes that results from high hydrostatic pressure (Yancey and Siebenaller, 1999; Yancey et al., 2001).

Other methylamines are known to counteract the effects of ammonia toxicity (Kloiber et al., 1988; Minana et al., 1996), salt (Dragolovich, 1994) and temperature stress (Krall et al., 1989; Nishiguchi and Somero, 1992) on protein function in a variety of organisms. Although TMAO may serve the functions attributed to it (i.e. it may be adaptive), it is not necessarily synthesized or accumulated as a specific adaptation to any of the stresses mentioned above. The source of TMAO in marine animals and the time course of its accumulation are not well characterized. Here, we present preliminary measurements of TMAO concentrations in a number of cephalopod species and review the distribution, biosynthesis and metabolism of methylamines in marine animals to assess their adaptive significance.

Methylamine synthesis

TMAO, like most methylamines, is derived from the trimethylammonium group of choline. Dietary choline may be oxidized to trimethylamine by bacteria in the gut of marine animals. The accumulation of TMA in rotting fish as a result of bacterial degradation of choline, as well as the reduction of TMAO to TMA, is responsible for their characteristic 'fishy' odor. TMA as a spoilage index for commercial fishes has been extensively discussed (Hebard et al., 1982; Sotelo and Rehbein, 2000). TMA is highly toxic (Marzo and Curti, 1997; Anthoni et al., 1991a,b) and so is, with at least one notable exception (Sanders and Childress, 1988), oxygenated within living animals to form TMAO (Fig. 1). This generally occurs within the digestive gland or liver *via* a monoxygenase enzyme, trimethylamine oxidase (Tmase) (Hebard et al., 1982). TMAO is then either transported to the tissues for accumulation as a compatible or 'counteracting' osmolyte or, more commonly, excreted. In humans, a mutation of the flavin-containing mono-oxygenase gene (FMO3) causes trimethylaminuria, a condition wherein individuals excrete TMA, rather than TMAO, along with a fishy body odor in urine, sweat, breath and other bodily excretions (Dolphin et al., 1997; Treacy et al., 1998).

There are, however, conflicting reports regarding the endogenous or exogenous (dietary) origin of TMAO in animals. Early reports suggested that invertebrates lacked Tmase activity (Baker et al., 1963). However, Tmase was later detected in copepods, indicating that dietary accumulation of TMAO at higher trophic levels does not necessarily rely on a cascade from phytoplankton production (Strøm, 1979). Trimethylamine oxidase activity has been detected in many, but not all, fishes (Baker et al., 1963). Among polar fishes, for example, those without detectable Tmase activity generally contained lower concentrations of TMAO than did species in which Tmase was detected (Raymond and DeVries, 1998). Goldstein et al. (1967) reported high concentrations of TMAO in dogfish (*Squalus acanthius*) tissues despite the reported absence of Tmase activity (Baker et al., 1963). They suggested that TMAO must be accumulated from the diet. However, Schlenk and Li-Schlenk (1994) later detected Tmase activity in dogfish as well as in the silky shark *Carcharhinus falciformis*. Nurse sharks also possess Tmase activity and are able to synthesize TMAO directly from choline *in vivo* without the aid of intestinal bacteria (Goldstein and Funkhouser, 1972). Conversion of choline to TMA and to TMAO does not, therefore, occur exclusively in the gut. However, the mechanism for conversion of choline to TMA other than by microbial oxidation is not known (Marzo and Curti, 1997).

Furthermore, fasted dogfish maintained stable TMAO concentrations over 41 days (Cohen et al., 1958). This result may be partly due to the active reabsorption of TMAO in the dogfish kidney (Cohen et al., 1958; Goldstein et al., 1967). Some sharks retain TMAO quite effectively, but substantial loss does occur such that maintenance of TMAO levels over 41 days seems unlikely without an endogenous source. Goldstein and Palatt (1974) found TMAO turnover rates of 4–14% per day in four different elasmobranch species, but excretion rates of less than 1% have been reported in some fishes (Agustsson and Strøm, 1981). Diets rich in TMA and choline have produced higher levels of TMAO in the muscles of some fish species but not in others (Goldstein et al., 1967; Agustsson and Strom, 1981). An endogenous source is suspected in many species, especially those that accumulate TMAO in high concentrations.

Endogenous choline supply is believed to limit the accumulation of glycine betaine (betaine), another common methylamine osmolyte, in euryhaline oysters (Pierce et al., 1997). Oyster mitochondria take up choline and convert it, *via*

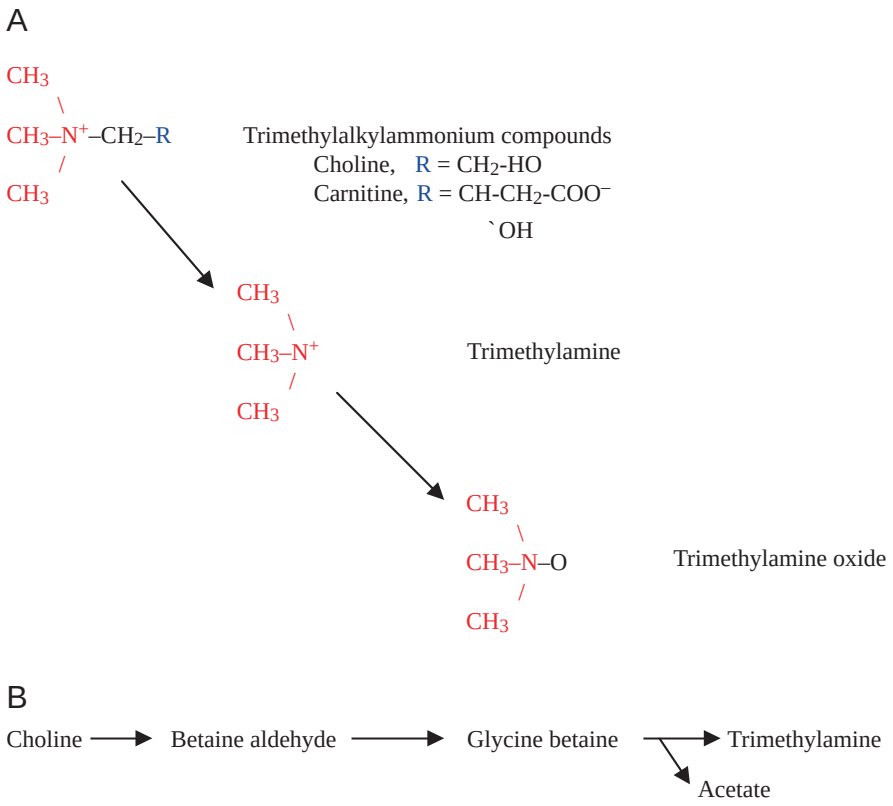


Fig. 1. Diagram showing the possible pathways for trimethylamine (TMA) and trimethylamine oxide (TMAO) production (only abbreviated pathways are drawn). (A) Trimethylalkylammonium compounds (e.g. choline) are degraded to TMA by intestinal microbes (Hebard et al. 1992). (B) Choline is taken up by mitochondria in some animals (Pierce et al., 1997) and converted to glycine betaine *via* betaine aldehyde. Betaine may subsequently be converted to TMA (Ballantyne, 1997). In both cases (A and B), TMA is oxidized by trimethylamine oxygenase to TMAO.

betaine aldehyde, to betaine (Fig. 1B). The enzymes involved in this transformation are well characterized (Dragolovich, 1994). Betaine could, if similarly produced in other species, subsequently be converted to TMA and TMAO (Fig. 1B). Some studies suggest that TMAO is a better counteracting solute than betaine.

Some plants produce methylamines, particularly betaine, reportedly for osmoregulation and protection against drought and salt-stress. A tremendous amount of research has been directed towards elucidating the pathways of betaine accumulation in plants in the hope of conferring drought-resistance to important commercial crops such as tobacco (Nuccio et al., 1998). Plant research may, thus, provide insight into the pathways and mechanisms of methylamine synthesis and storage in marine animals. Several pathways for the production of choline in plants have been identified. Drought-resistant plants, such as chenopods (e.g. spinach and sugar beet), are adapted for direct production of choline from ethanolamine (Hanson and Rhodes, 1983; Summers and Weretilnyk, 1993; Weretilnyk et al., 1995). Radioactively labeled ethanolamine in spinach is recovered primarily as betaine. In contrast, labeled ethanolamine in wheat and barley is recovered primarily in phospholipid (Hitz et al., 1981; McDonnell and Wyn Jones, 1988) (Fig. 2).

Wheat does appear to accumulate betaine, but only through phosphatidylcholine hydrolysis (Fig. 2B). In wheat and barley leaves, phosphatidylcholine is hydrolyzed to diacylglycerol and choline. A similar pathway is suspected for oysters and horseshoe crabs, which convert choline to betaine as well (Pierce et al., 1997). Diacylglycerol, produced *via* phosphatidylcholine hydrolysis in wheat plants, is subsequently converted to monogalactosyldiacylglycerol, an essential glycolipid in photosynthetic membranes. Free choline is converted to betaine (McDonnell and Wyn Jones, 1988). In drought-resistant chenopods, betaine accumulation does not depend on phosphatidylcholine hydrolysis, a fact that may result in their

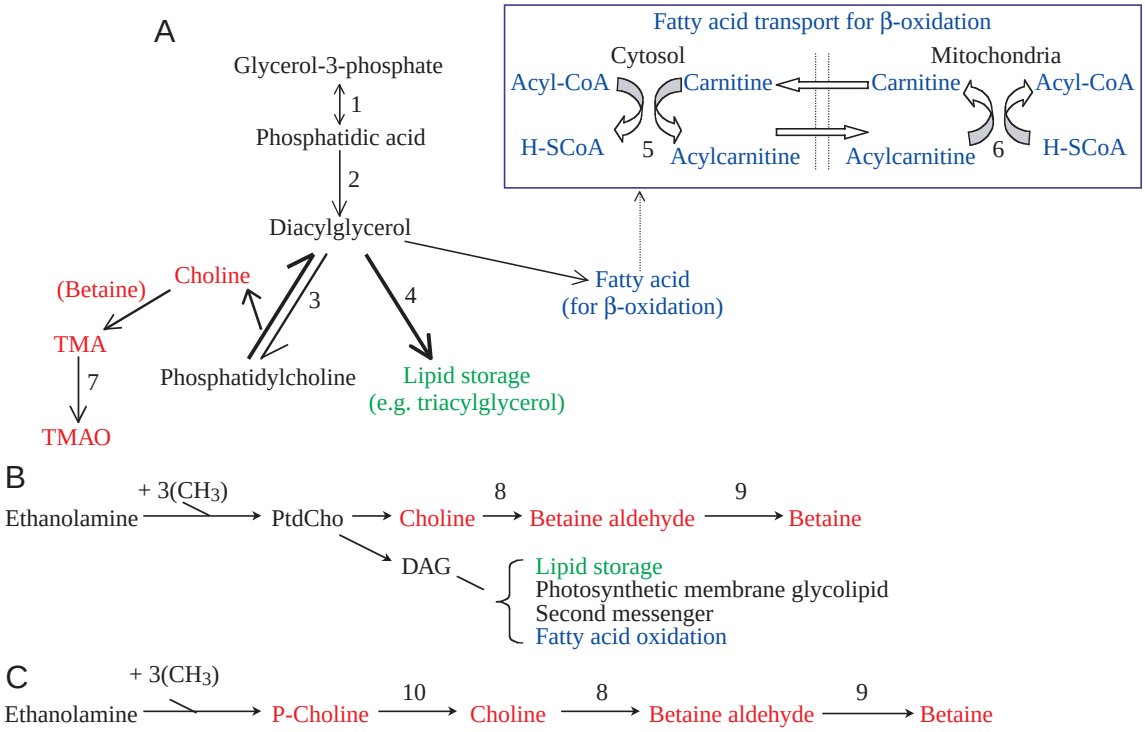


Fig. 2. (A) Diagram of the glycerolphosphate pathway and phosphatidylcholine synthesis as found in both animals and plants. The bold arrows indicate hypothesized pathways resulting in simultaneous accumulation of acylglycerols and trimethylamine oxide (TMAO) (only abbreviated pathways are drawn). Diacylglycerol (DAG), formed from the derivative glycerol-3-phosphate (DAG), can be shunted towards either acylglycerol (lipid) storage (e.g. triacylglycerol) or phosphatidylcholine (PtdCho) synthesis. The final step in the phosphatidylcholine pathway is reversible. The back reaction may occur to a significant extent so that diacylglycerol is formed from phosphatidylcholine and subsequently stored for seasonal or reproductive energy reserves (see Gur and Harwood, 1991). Choline, thus released, is oxidized to TMAO and either stored or excreted. (B) In some plants, PtdCho is produced by methylation of ethanolamine and may subsequently be hydrolysed for release of DAG and choline. DAG in plants is important during growth for the formation of photosynthetic membrane glycolipids. Choline, thus released, is oxidized to glycine betaine (Hitz et al. 1981). (C) In chenopods (e.g. spinach and sugarbeet), choline is synthesized directly from ethanolamine and, thus, appears to be a specific adaptation for glycine betaine accumulation during drought and salt-stress (Weretilnyke et al., 1995). The enzymes involved in the pathways illustrated are numbered: (1) glycerol-3-phosphate (G3P) is converted to phosphatidic acid by the successive actions of G3P acyltransferase and 1-acylglycerol-3-phosphate acyltransferase; (2) phosphatidic acid phosphatase; (3) 1,2-diacylglycerol:choline phosphotransferase; (4) diacylglycerol acyltransferase; (5,6) carnitine palmitoyl transferases I and II; (7) trimethylamine oxygenase; (8) choline monoxygenase; (9) betaine aldehyde dehydrogenase; (10) P-choline phosphatase. TMA, trimethylamine; P-choline, phosphocholine.

greater relative capacity for betaine accumulation and their greater salt tolerance (McDonnell and Wyn Jones, 1988).

Phosphatidylcholine hydrolysis is also responsible for diacylglycerol production in plants that store large quantities of triacylglycerols in their seeds (Gur and Harwood, 1991). Triacylglycerol sometimes constitutes as much as 80 % of the dry mass of the seeds. Triacylglycerols are also among the most common form of energy reserve in animals, but diacylglycerol ethers are also frequently stored. Many animals store acylglycerols as metabolic fuel reserves that can, during starvation, migration, reproduction or egg development, be mobilized and oxidized to drive metabolic processes. Phosphatidylcholine hydrolysis, which is important during accumulation of acylglycerols, may serve as an endogenous source of choline, resulting in TMAO accumulation in animals. The enzymes that are required for phosphatidylcholine hydrolysis are apparently well conserved, having been found in mammals, molluscs and arthropods as well as the plants mentioned above (Anfuso et al., 1995).

Diacylglycerol and phosphatidylcholine

The predominant pathway for the biosynthesis of triacylglycerol and diacylglycerol ether is the glycerol phosphate pathway. Glycerol phosphate, a derivative of glycolysis, is converted to diacylglycerol *via* phosphatidic acid (Fig. 2). Diacylglycerol represents a branchpoint where diacylglycerol can be either channeled into phospholipid synthesis (i.e. phosphatidylcholine) or acylated to form triacylglycerols. The final step towards the synthesis of phosphatidylcholine is catalyzed by 1,2-diacylglycerol:choline phosphotransferase (CPT). CPT activity governs the partitioning of diacylglycerol into either phosphatidylcholine or acyglycerol pools (Jackowski et al., 2000). The back reaction of CPT can occur to a significant extent so that diacylglycerol is formed from phosphatidylcholine, releasing free choline (for a review, see Gur and Harwood, 1991).

In animals, diacylglycerol released from phosphatidylcholine hydrolysis is an important second messenger (Billah and Anthes, 1990; Wakelam et al., 1993). In fact, during hypo-osmotic cell volume regulation, cell swelling results in membrane turnover and phosphatidylcholine hydrolysis, resulting in the formation of diacylglycerol. Diacylglycerol activates phosphokinase C which, in turn, stimulates the release of osmolytes in a range of organisms from sharks (Musch and Goldstein, 1990) to algae (Thompson, 1994). Cell volume regulation *via* phosphatidylcholine hydrolysis under hyperosmotic stress (i.e. dehydration) could contribute to the release of free choline and subsequent betaine or TMAO accumulation in some animals (although some mechanism would be required to prevent diacylglycerol from stimulating the release of osmolytes in this case). For example, TMAO was shown to accumulate during dehydration in frog gastronemius muscle (Wray and Wilkie, 1995), although the authors postulate that TMAO accumulated to counteract increased urea concentrations. Choline produced *via* phosphatidylcholine hydrolysis has no known function other than the synthesis of acetylcholine in neural tissue (Billah and Anthes, 1990). We propose that phosphatidylcholine hydrolysis *via* the back reaction of CPT may provide an endogenous source of choline for TMAO synthesis in animals.

Hydrolysis of dietary phosphatidylcholine by phospholipases C and D may also be an important source of free choline for TMAO synthesis (Wakelam et al., 1993). Hydrolysis of labeled phosphatidylcholine by phospholipase C in the gut of larvae of the dragonfly *Aeshna cyanea* resulted in recovery of labeled products in various forms, including acylglycerols and glycine betaine (Weiher and Komnick, 1997).

Ordinarily, phospholipid synthesis takes precedence over triacylglycerol synthesis in plants and animals when the demand for accumulation of fuel stores is low. This ensures the maintenance of membrane turnover, an essential physiological process. However, during periods when storage of fuel is essential, such as preparation for seasonal reductions

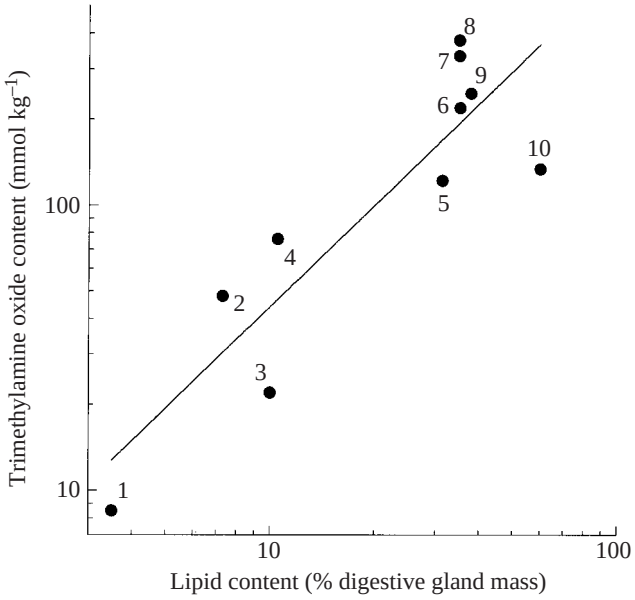


Fig. 3. Trimethylamine oxide content (y, mmol kg^{-1}; see Fig. 4) in mantle muscle tissue is significantly correlated with digestive gland lipid content (x) in cephalopods (y=2.90$x^{1.18}$, r=0.59, P<0.05). We were unable to analyze trimethylamine oxide (TMAO) content as a function of total lipid content because of the variable presentation of lipid data in the literature. Lipid data were taken from Blanchier and Boucaud-Camou (1984), Hayashi (1989, 1996), Hayashi and Kawasaki (1985), Kristensen (1984), Phillips et al. (2001), Piatkowski and Hagen (1994), Pollero and Iribarne (1988) and Semmens (1998). In many cases, lipid and TMAO data were taken from different species, and possibly different maturity stages, of the same genus. (1) *Octopus*; (2) *Loligo*; (3) *Galiteuthis*; (4) *Sepia*; (5) *Illex*; (6) *Berryteuthis*; (7) *Moroteuthis*; (8) *Gonatopsis*; (9) *Todarodes*; (10) *Gonatus*. There are conflicting reports regarding the digestive gland lipid content of *Thysanoteuthis*, a squid that also contains high TMAO concentrations (Hayashi, 1996; Yuneva et al., 1994). Many of the genera plotted here are closely related to each other (see Fig. 4). Therefore, phylogenetic independence of the data should not be assumed.

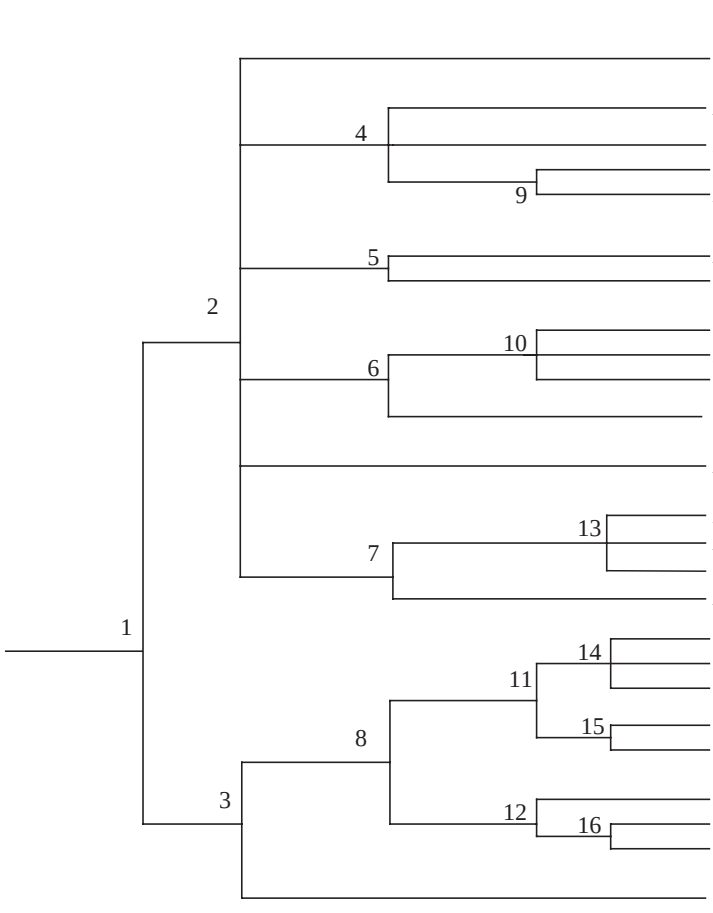
in productivity, migrations or reproductive events, diacylglycerol is preferentially channeled towards triacylglycerol synthesis or is converted to diacylglycerol ether. In at least one case, phosphatidylcholine itself is used as a seasonal lipid reserve (Hagen et al., 1996). During such periods, diacylglycerol production may be enhanced by phosphatidylcholine hydrolysis *via* phospholipases or through the back reaction of CPT. The regulation of the enzymes involved in these processes is poorly understood, but probably involves hormonal changes associated with ontogenetic, seasonal and reproductive events.

Acylglycerol and TMAO: correlation

A general correlation exists between the concentration of TMAO (and betaine) in muscle tissue and lipid, particularly diacylglycerol ethers and triacylglycerols, levels in the bodies of marine animals. TMAO and lipid concentration both appear to be correlated with habitat depth, latitude, season, lifestyle (e.g. benthic *versus* pelagic) and ontogeny or size (for reviews, see Hebard et al., 1982; Sargent, 1976, 1989). Both TMAO and lipid also appear conspicuously within the same phylogenetic groups.

Cod (gadiform teleosts) are sought commercially for their abundant liver oil and have high concentrations of TMAO in their muscle tissue (Gillett et al., 1997; Agustsson and Strøm, 1981). Among Antarctic fishes, *Dissostichus* sp. has the highest concentrations of both body and liver lipids (Eastman, 1988; Friedrich and Hagen, 1994) and TMAO (Raymond and DeVries, 1998). Elasmobranchs (sharks) generally contain high levels of both liver lipids (Baldridge, 1970; Bone and Roberts, 1969) and TMAO (Withers et al., 1994). For example, *Squalus* sp. and *Somniosus* sp. have among the highest reported TMAO levels (Anthoni et al., 1991a; Goldstein et al., 1967) and are sought commercially for the high concentrations of diacylglycerol ether (DAGE) in their livers (Hallgren and Stallberg, 1974; Kang et al., 1997). *Somniosus microcephalus* has even been implicated in trimethylamine poisoning (Anthoni et al., 1991a). Holocephalans, a generally deep-living subclass of Chondrichthyes, also contain large quantities of methylamines (both betaine and TMAO) and lipid (Hayashi and Takagi, 1980; Hebard et al., 1982; Bedford et al., 1998).

Among invertebrates, the correlation between TMAO and acylglycerol levels is most notable in the cephalopods (Fig. 3), especially in the deep-sea squid families Onychoteuthidae



Species	*N*	MDO	CD	TMAO	Corrected
Galiteuthis phyllura	2	10	500	16, 28	
Loligo opalescens	-	10	10	(48)	
Bathyteuthis berryi	1	800	800	108	
Sepia officinalis	-	10	10	(48-105)	
Rossia pacifica	1	10	200	16	
Mastigoteuthis famelica	1	500	1000	67	102
Chiroteuthis calyx	2	300	750, 950	27, 68	57–142
Gonatus onyx	4	100	100–700	48–152	
Berryteuthis magister	1	100	(1800)	(219)	
Gonatopsis borealis	1	100	(2850)	(377)	
Thysanoteuthis rhombus	1	10	-	(183-203)	
Histioteuthis heteropsis	3	150	500	89-127	148–211
Illex illecebrosus	2	10	10	100, 144	
Dosidicus gigas	1	10	10	175	
Todarodes pacificus	-	10	10	(157-335)	
Moroteuthis robusta	1	100	(2850)	(333)	
Octopus dofleini	-	10	10	(29)	
Octopus bimaculoides	1	10	10	8.5	
Octopus vulgaris	-	10	10	(8.5)	
Graneledone sp.	1	1000	2000	24.8	
Octopus californicus	1	200	500	35.5	
Japetella heathi	2	600	1000	0, 3	
Opisthoteuthis sp.	1	200	300	10	
Cirrothauma murrayi	1	2000	2200	41	
Vampyroteuthis infernalis	1	600	700	38	

Fig. 4. Mantle muscle trimethylamine oxide (TMAO, mmol kg^{-1}) measured according to Wekell and Barnett (1991), minimum depth of occurrence (MDO, m) and capture depth (CD, m) of cephalopods listed according to their phylogenetic associations (see Carlini and Graves, 1999). Values are corrected for the dilution of tissue with extracellular ammonium concentrations in some species (see text). Nodes are numbered for reference in the text. Numbers in parentheses are from Hebard et al. (1982) or Kelly and Yancey (1999).

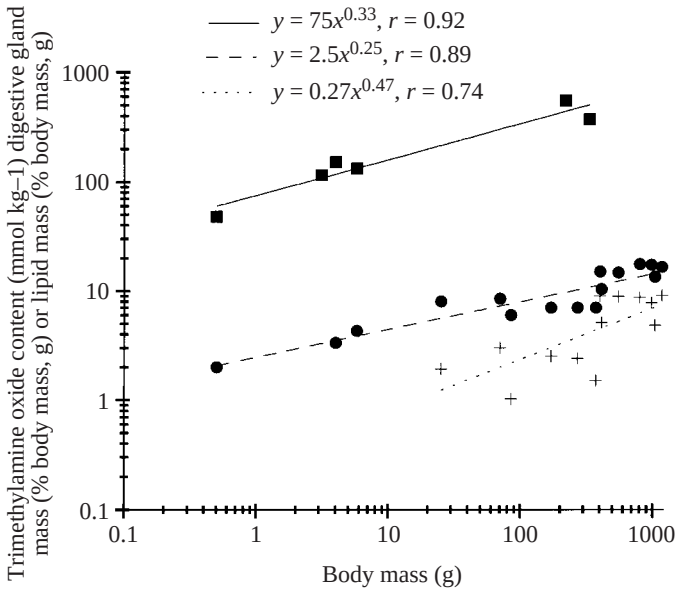


Fig. 5. Mantle muscle trimethylamine oxide (TMAO) content (mmol kg^{-1}; squares) increases (b=0.33) in proportion to digestive gland mass (% body mass; circles; b=0.25) and lipid mass (% body mass; plus signs; b=0.47) through ontogeny (size; g) in gonatid squid (Cephalopoda). The data for TMAO content are from Kelly and Yancey (1999) and B. A. Seibel (unpublished results). Body masses for the TMAO scaling analysis (squares) for *Gonatopsis borealis* and *Berryteuthis magister* were estimated at the mean adult body mass for each species (Hayashi, 1989; Hayashi and Yamamoto, 1987). Digestive gland and lipid masses were taken from various sources: *Gonatus onyx* (B. A. Seibel, personal observation), *Gonatus fabricii* (Arkhipkin and Bjorke, 1999), *Gonatopsis borealis* (Hayashi, 1989) and *Berryteuthis magister* (Hayashi and Yamamoto, 1987). All species appear to fall on the same scaling line despite differences in maximum body sizes. The apparent correlation between lipid content (or digestive gland mass) and TMAO content in gonatid squid is hypothesized to result from the requirement for phosphatidyl choline hydrolysis to produce diacylglycerol for lipid storage. The choline thus released is oxidized to TMAO.

(*Moroteuthis robusta*) (Fig. 4; node 7) and Gonatidae (Fig. 5) (Fig. 4, node 10). While most cephalopods studied to date have very low lipid concentrations (O'Dor and Webber, 1986) and little ability to metabolize lipids (Hochachka, 1994), gonatid and onychoteuthid squids have acylglycerol contents as high as 25% of their body mass (Hayashi and Kawasaki, 1985; Hayashi et al., 1990; Phillips et al., 2001). These two families also contain the highest TMAO concentrations among cephalopods (Fig. 4) (Hebard et al., 1982; Kelly and Yancey, 1999). Some gonatid squids apparently contain high concentrations of glycine betaine as well (Shirai et al., 1997).

Both gonatid and onychoteuthid squid are known to undertake ontogenetic vertical migrations whereby successive developmental stages occupy progressively greater depths. This migration is believed to end at depths greater than 1500 m, at which spawning and, at least in some cases, egg-brooding take place (Jackson and Mladenov, 1994; Arkhipkin and Bjorke, 1999; Seibel et al., 2000). The high lipid content in gonatid squids accumulates throughout their life and is thought to fuel an extended egg-brooding period (Seibel et al., 2000). The high TMAO values reported for gonatid squids were found in adult specimens. Preliminary measurements suggest that the concentration of TMAO is much lower in smaller individuals of the species (Fig. 5) and, as for lipids, that TMAO accumulates through ontogeny.

Given the ontogenetic descent to great depths undertaken by gonatid squids, an ontogenetic increase in TMAO concentration is consistent with the hypothesis that TMAO counteracts the effects of high hydrostatic pressure on proteins (Kelly and Yancey, 1999). However, values for other cephalopods (Hebard et al., 1982) (Fig. 4) suggest that TMAO content is not related to depth (although data on glycine betaine and other methylamines would be helpful to analyze this hypothesis fully). For example, ommastrephids are predominantly shallow-living squid that contain large amounts of TMAO (100–335 mmol l^{-1}; Fig. 4, node 13) (Hebard et al., 1982). Ommastrephids accumulate lipid, up to 6% of body mass, which is thought to fuel a reproductive horizontal migration (Takahashi, 1960; Clarke et al., 1994). Incidentally, this family apparently has a 'different' smell from other squids and is known to cause allergic reactions in some people (Vecchione, 1994). Both phenomena are perhaps related to trimethylamine.

Bathyteuthidae and Histioteuthidae, midwater squid families known to have large oily livers, were also found to accumulate TMAO (Fig. 4). Furthermore, no relationship between TMAO content and minimum or capture depth was found for octopods over a wide depth range (Fig. 4, node 8). Incirrate octopods (Fig. 4, node 11) have low TMAO levels at all depths. Cirrate octopods (Fig. 4, node 16) also have fairly low levels of TMAO, but slightly higher levels of betaine (>50 mmol kg^{-1}) (Yin and Yancey, 2000). The few shallow-living octopods measured have had very low lipid contents (O'Dor and Webber, 1986; Pollero and Iribarne, 1988). Lipid levels have not been measured in cirrate octopods. The general increase in TMAO concentration with depth observed in a variety of animals (Kelly and Yancey, 1999) may be related to the tendency for deep-living species to accumulate lipids (e.g. Childress and Nygaard, 1974).

Some midwater squid accumulate ammonium (approximately 300 mmol kg^{-1}) in their tissues for buoyancy. Although previous methods measuring ammonium did not distinguish between ammonium and methylamines (Sanders and Childress, 1988), our recent analysis using high-performance liquid chromatography confirmed the existence of high ammonium concentrations in most midwater squid species (B. A. Seibel, unpublished data). Such species appear to have special vacuolated tissue in which ammonium is sequestered, presumably out of contact with intracellular macromolecules (Voight et al., 1994). If this sequestration is incomplete, however, TMAO could be required to counteract the toxic effects of ammonium on enzymes in ammoniacal squid. Preliminary measurements suggest that ammonium does depress the activity of octopine dehydrogenase from

Histioteuthis heteropsis muscle tissue. However, TMAO did not effectively counteract this depression (B. A. Seibel, unpublished data). Furthermore, most species with high TMAO concentrations are negatively buoyant and do not accumulate ammonium. In the case of gonatids, the ontogenetic stages with high TMAO values also have lipid contents sufficient to provide neutral buoyancy and do not require ammonium or TMAO to provide lift.

It should be pointed out that cirrate and some pelagic incirrate octopods have extensive extracellular gelatinous tissue. We made every effort to remove the external gelatinous tissue, but some dilution of the TMAO content is expected in these species. Inclusion of the extracellular ammonium vacuoles in the analyzed tissue of some midwater squid certainly diluted the tissue homogenates as well. Assuming that all ammonium in such species is contained as a 500 mmol l^{-1} solution in extracellular vacuoles (Voight et al., 1994) and that all TMAO measured is intracellular, we have corrected these values (Fig. 4) using measured ammonium and protein contents (B. A. Seibel, unpublished data). *Bathyteuthis berryi* does not possess ammonium as previously reported, but does contain high concentrations of an as yet unidentified nitrogenous cation, possibly TMA (B. A. Seibel, unpublished data) (Voight et al., 1994). We believe that this cation is intracellular and so no correction to the TMAO value in Fig. 4 has been applied to this species. *Galiteuthis phyllura* accumulates ammonium in a specialized coelomic chamber well out of contact with muscle tissue. No correction was applied to this species either. TMAO was not found in the coelomic fluid of *Galiteuthis phyllura*.

We recently measured high TMAO concentrations in *Clione antarctica* (112 mmol kg^{-1}) (B. A. Seibel, unpublished data), a shallow-living Antarctic pteropod mollusc known to store high concentrations of DAGE within its body (Kattner et al., 1998; Phleger et al., 1997). *Clione antarctica* feeds exclusively on the thecosomatous pteropod *Limacina helicina*, which accumulates large quantities of dimethylsulfoniopropionate (DMSP) in its body (Levasseur et al., 1994) directly from phytoplankton in its diet. DMSP is the sulphidic analog of the nitrogenous glycine betaine and may be the precursor of the sulfonium analogue of phosphatidylcholine in some phytoplankton (Kates and Volcani, 1996). Like trimethylamine oxide, DMSP is known to confer some protection against osmotic and temperature stress in phytoplankton (Nishiguchi and Somero, 1992). Fishes that feed on *Limacina helicina* are often inflicted with ‘blackberry feed’, a foul-smelling and aesthetically displeasing condition resulting from the breakdown of DMSP to dimethylsulfide, subsequently accumulated in the fish’s tissues (Levasseur et al., 1994). Although the TMAO measured in *Clione antarctica* may be related to the large lipid stores, some connection to the DMSP concentrations in *Limacina helicina* cannot be ruled out. The synthetic pathways of DMSP and betaine are linked (Mulholland and Otte, 2000). Trimethylamine oxide levels in *Limacina helicina* have not been measured.

Time course of methylamine accumulation

Although some groups, such as elasmobranchs, have specific adaptations for retention of TMAO to counteract urea toxicity, other groups may simply allow TMAO to accumulate while it is available and thus reap the benefits of its compatibility and protein-stabilizing attributes. The time course of glycine betaine accumulation in wheat plants during, and subsequent to, periods of leaf expansion is consistent with this explanation (Hitz et al., 1981; McDonnell and Wyn Jones, 1988). The most rapid betaine accumulation in the leaves of unstressed wheat plants, and initially in those of salt-stressed wheat plants, is at the time of greatest leaf expansion and, consequently, membrane development and membrane lipid (e.g. phosphatidylcholine) turnover. When growth (glycolypid biosynthesis) plateaus, betaine concentrations slowly decline (McDonnell and Wyn Jones, 1988).

The relatively low TMAO concentrations (48 mmol kg^{-1}) that we recently measured in a gonatid squid that was brooding an egg mass are also consistent with this model (B. A. Seibel, unpublished data). This squid was using, rather than storing, lipid and had lost the TMAO that it had presumably accumulated prior to spawning (Fig. 5). Dehydrated frog muscle initially accumulated methylamines in concert with rising urea concentrations (Wray and Wilkie, 1995). However, urea concentrations continued to rise with continued dehydration, while methylamine levels reached a plateau.

Adaptive significance of methylamines

The role of phosphatidylcholine hydrolysis in the production, as well as the time course of accumulation, of betaine led Hitz et al. (1981) to question the adaptive significance of betaine accumulation in wheat and barley. Betaine accumulation may only be a side-effect of accelerated turnover of phospholipid head groups during stress and not a specific adaptation to stress from which some benefit accrues to the stressed leaf (Hitz et al., 1981). In oysters, betaine synthesis and accumulation may be a consequence of cell membrane restructuring during cell volume changes resulting in phosphatidylcholine hydrolysis (cf. Dragolovich, 1994; Musch and Goldstein, 1990). Similarly the temporary accumulation of trimethylamines in frog muscle during dehydration may also reflect membrane turnover during hyperosmotic cell volume regulation (see Wray and Wilkie, 1995) and not necessarily a response to increased urea concentrations. The adaptive significance of DMSP accumulation in algal cells has also been called into question by Stefels (2000), who noted that changes in the concentration of DMSP in algal cells upon salt-stress are the result of metabolic changes rather than active regulatory mechanisms. He suggested that DMSP may be considered as a compatible solute, but that it is not osmoticum in the strict sense of being responsible for osmotic balance. Perhaps TMAO accumulation in marine animals reflects, in part, the requirements for diacylglycerol for lipid storage.

Fatty liver in sharks

In mammals, dietary choline deficiency prevents phosphatidylcholine synthesis and may leave excess diacylglycerol, produced in the glycerol phosphate pathway, to be channeled towards triacylglycerol for storage in the liver (Fig. 2). This condition, known as 'fatty liver', may be a chronic condition in sharks. The requirements for TMAO accumulation to counteract urea toxicity may limit the availability of choline for phosphatidylcholine synthesis. As a consequence, lipid may accumulate in the liver. The loss of extra-hepatic fatty acid oxidation may further contribute to fatty liver in sharks. Freshwater stingrays alone among elasmobranchs possess the ability to oxidize fatty acids extra-hepatically. Because they have no need to accumulate urea as an osmolyte, freshwater stingrays also do not accumulate TMAO in their tissues. This led Ballantyne and Moon (1986) to postulate a relationship between extra-hepatic β-oxidation and the absence of urea and TMAO accumulation. One possible cause is competition for carnitine which, like choline, may be oxidized to TMAO (Marzo and Curti, 1997). Eliminating β-oxidation may allow all available carnitine to be converted to TMAO. By limiting β-oxidation in non-hepatic tissue, sharks may also decrease the competition for diacylglycerol for lipid accumulation in the liver. Limited extra-hepatic fatty acid oxidation capacity in squid (Ballantyne et al., 1981) may similarly preadapt them for seasonal and ontogenetic acylglycerol accumulation. However, the only genus for which the capacity for fatty acid oxidation has been measured (*Loligo*) does not appear to accumulate lipid or TMAO. The lipid-rich livers of sharks are generally attributed a buoyancy role (Malins and Barone, 1970; Phleger, 1998; Wetherbee and Nichols, 2000). Although the low-density oils, especially DAGE and squalene, do provide lift, they may simply be a beneficial end-product of TMAO production in sharks.

Although this 'fatty liver' scenario could, in theory, also apply to animal groups other than elasmobranchs, our inability to identify a cellular perturbant that consistently explains the distribution of TMAO in cephalopods and other marine animals causes us to reject this possibility. Among marine animals, methylamine accumulation as an adaptation for macromolecular stabilization has, in our opinion, been convincingly demonstrated only in sharks.

Fatty liver in mammals often results in cancerous tumor production (Goshal and Farber, 1984; Locker et al., 1986). It may be no coincidence that shark livers (and squid digestive glands for that matter) also contain large quantities of squalamine, derived from squaline lipids and/or diacylglycerol ethers. Both squalamine and diacylglycerol ethers have recently been shown to inhibit tumor development by limiting vascular growth (Sills et al., 1998).

Concluding remarks

We suggest that hydrolysis of phosphatidylcholine contributes to TMAO accumulation in many marine organisms. TMAO may simply be accumulated as a compatible solute in quantities reflecting the amount of lipid stored in the body. Conversely, among sharks, the requirement for TMAO accumulation may deplete available choline levels, thus limiting the production of phosphatidylcholine and shunting excess diacylglycerol, produced in the glycerol phosphate pathway, towards storage in the liver. Sharks alone among marine animals may possess specific adaptations for retention, and possibly for production *via* centralization of fatty-acid oxidation, of TMAO. We do not rule out the possibility that TMAO is strongly selected for its protein-stabilizing attributes in some other animal groups, possibly resulting in lipid accumulation as a metabolic byproduct. It is also possible that that diacylglycerol and TMAO levels are linked, as we have proposed, but that the maintenance of high TMAO levels reflects retention adaptations in response to some cellular perturbant. However, no obvious protein-destabilizing agent has been identified in the cephalopods examined here that would warrant the observed accumulation of TMAO. Because of the paucity of data on methylamines other than TMAO, we hesitate to rule out the possibility that hydrostatic pressure selects for high methylamine concentrations in deep-sea organisms. We also cannot rule out the possibility that pathways for the accumulation of lipid and TMAO are not coupled in some cases. However, we feel that the link between phosphatidylcholine hydrolysis and trimethylamine oxide accumulation should be considered a competing hypothesis worthy of further investigation.

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