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A COMPARATIVE STUDY OF TRIMETHYLAMINE-*N*-OXIDE BIOSYNTHESIS

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SUMMARY

A broad spectrum of species of plants and animals have been examined for their ability to convert trimethylamine to its *N*-oxide. Of the animal species studied, and under the conditions used in this investigation, trimethylamine-*N*-oxide synthesis from trimethylamine seems generally to be restricted to the vertebrates. Within this group at least one example of each class has been shown competent to carry out the reaction. An exhaustive examination of fishes has shown elasmobranches, and both fresh- and salt-water teleosts to be active in trimethylamine-*N*-oxide biosynthesis; however, many species of fishes did not given detectable trimethylamine-*N*-oxide synthesis under the conditions of assay. Although all attempts to demonstrate trimethylamine-*N*-oxide synthesis in plants *in vitro* were negative, suggestive evidence was obtained that certain plants can carry out this synthesis *in vivo*.

INTRODUCTION

In the preliminary phases of a study of the general mechanism by which biological systems effect molecular oxygenations, our attention was drawn to the biosynthesis of TMAO as a simple model system upon which to carry out our investigation. Subsequently we have found[1] that the properties of a TMA-oxidizing enzyme of hog liver are those of a mixed function oxidase (as described by MASON[2]). Thus, TMA is only oxidized to TMAO in TPNH and molecular oxygen are supplied to the hog-liver microsomal system.

Although a number of physiological functions have been ascribed to TMAO[3], little is known of its biosynthetic origin. The finding of a TMA-oxidizing enzyme in hog liver and the probability that it catalyzes the final step of TMAO biosynthesis suggested the present study to determine the distribution of the enzyme in other species. A wide variety of tissues from plant and animal sources have been assayed. Evidence presented in this report demonstrating the ability of many organisms to perform the synthesis of TMAO gives permissive support to the several physiological roles which have been proposed for TMAO.

Abbreviations: TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide.

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EXPERIMENTAL

The capacity of tissues to oxidize TMA to TMAO was determined by preparing a homogenate in 0.05 M potassium pyrophosphate buffer (pH 8.2), incubating this preparation with [^{14}C]TMA in the presence of a TPNH-generating system for 20 min, deproteinizing with perchloric acid, absorbing any [^{14}C]TMAO formed on to Dowex-50 (H^+), eluting with 5 % aqueous ammonia and counting an evaporated aliquot at infinite thinness. Details of this method have been published[1]. Some results are quoted for which the earlier method of BAKER AND CHAYKIN[4] was employed. In this case yields of [^{14}C]TMAO are given in terms of counts/min, as the method does not allow absolute values to be determined.

The methods of KOUKOL AND CONN[5] were used for germinating seeds, growing seedlings (3–7 days old), administering ^{14}C-labelled substrate to intact plant tissues, and extracting products in a form suitable for chromatography. TMAO was qualitatively identified by paper chromatography on Whatman No. 1 paper in a solvent system composed of *n*-butanol–glacial acetic acid–water (60:15:25); and quantitatively determined as described above. For studies *in vivo*, 3–7 day old seedlings were disrupted either by the preparation of an acetone powder or by grinding with a mortar and pestle in the presence of liquid nitrogen. The extract obtained on treatment of each gram of disrupted plant material with 2.5 ml of 0.05 M potassium pyrophosphate buffer (pH 7.9) was assayed for TMAO-synthetic capacity.

RESULTS AND DISCUSSION

In the present study, reaction conditions known to be optimal for TMA oxidation by hog-liver microsomes[1] have been employed to assay for TMAO synthesis in the tissues of other species. The possibility remains that in those species with no demonstrable TMA-oxidizing capacity, TMAO is synthesized *in vivo* under different reaction conditions or by another pathway. Evidence was provided[1] that the product of hog-liver microsome-catalyzed TMA oxidation is TMAO, but in the present work the assumption has been made that the only compound metabolically derivable from TMA which retains its basic properties and is non-volatile under basic conditions is TMAO.

The data presented in Tables I–III, when considered in the frame work of the limitations of the methodology employed, indicate that amongst the animal species investigated the conversion of TMA to TMAO is restricted to the vertebrates. All mammalian livers assayed (rat, guinea-pig, sheep, hog and rabbit) were active (Table I). Somewhat lower activity was also present in kidney homogenates.

It has been suggested that in mammals TMAO is a detoxication product of the TMA produced from choline by the bacterial flora of the gut[6]. Since it is generally considered that the liver and kidneys serve as the major sites of mammalian detoxication processes, it is not surprising to find these tissues active in the conversion of TMA to its *N*-oxide. The fact that lung is also quite competent in TMAO biosynthesis, although unexpected, becomes acceptable teleologically speaking when one considers that the atmosphere with which the lungs are in contact can contain a variety of potentially toxic amines, including TMA. The lung could then be thought of as the site of the organisms first line of defense against air borne toxic chemical agents, in much the same sense as the liver protects the organism from similar agents passing the gut.

The possession by chicken, turtle, necturis and frog of the TMA-oxidizing enzyme (Table II) might likewise indicate a detoxication mechanism. Of particular interest is the finding that the bullfrog tadpole liver did not support the oxidation of TMA, whereas the adult liver was active. If the bullfrog enzyme, like that of hog, rabbit, and rat liver, is microsomal, correlation of changes in microsomal structure and enzymic activity during metamorphosis becomes possible.

TABLE I

TMAO BIOSYNTHESIS IN MAMMALS

Source of enzyme	TMAO synthesized		
	Counts/min*	(μmoles/h/g of tissue**) A	B
Guinea-pig liver		14	
Rat liver		13	
Sheep liver	3208		
Hog liver	969		3.6
Hog kidney	171		
Hog heart	38		
Rabbit liver	1801	11	26
Rabbit kidney	369		4.2
Rabbit lung	189		7.3
Rabbit skeletal muscle	64		<1.0
Rabbit brain	94		<1.0
Rabbit spleen	90		
Rabbit testis	72		
Rabbit ovary			<1.0
Rabbit adrenal	76		
Rabbit blood	120		

* Results obtained by the method of BAKER AND CHAIKIN[4].

** Assay reaction mixture contained: A, 0.207 μmole [^{14}C]TMA·HCl of specific activity 207 μC/mmole; B, 1.97 μmoles [^{14}C]TMA·HCl of specific activity 21.7 μC/mmole.

The data in Table II also indicate that both fresh- and salt-water fish can convert TMA to TMAO. In elasmobranchii 100–120 mmoles/l of TMAO are present in blood[7], and it has been suggested that TMAO plays an important part in osmoregulation in these fish[3]. Synthesis of TMAO is not essential for this role, as TMAO may be of dietary origin. Osmoregulation could perhaps result from the control of its excretion[8] coupled with the reversible binding of TMAO molecules to a large molecule, such as a protein. Our results show that shark may synthesize TMAO, as its liver has the capacity to oxidize TMA. Of the dogfish livers assayed one was active, but others would not support TMA oxidation. Livers of the other two elasmobranch species tested (skate and electric ray) were inactive. Particularly if TMAO is important in osmoregulation, its synthesis may be dependent upon the working of a regulatory system. Then in our assay of liver homogenates, TMA-oxidizing activity would be found when, by chance, the fish is caught at a time of replenishing its TMAO reserve.

The Teleostei contain much lower levels of TMAO and this is mainly present in muscle[3]; so an osmoregulatory function is less likely in these types. Nevertheless the TMAO content of anadromous fish increases markedly on transfer from fresh to salt

TABLE II

TMAO BIOSYNTHESIS IN OTHER VERTEBRATES

(f) indicates a frozen sample of liver assayed; all other samples were of fresh tissue taken for assay immediately after sacrificing the animal.

Source of enzyme	*TMAO synthesized* (μmoles/h/g of tissue)*	
	A	*B*
Birds		
Chicken liver	9.3	
Reptiles		
Turtle liver	1.7	
Amphibia		
Necturis liver	3.9	
Fog liver (*Rana pipiens*) (adult)	2.7	
Bullfrog (*Rana catesbeiana*) liver (adult)		6.5
Bullfrog (*Rana catesbeiana*) liver (tadpole)		<0.1
Fishes (salt water)		
(a) Elasmobranchii		
Dogfish (*Squalus acanthias*, L.) liver**	<1	
Shark (*Mustelus californicus*, Gill) liver (f)		17.1
Skate (*Raja binoculata*, Girard) liver	<1	<0.1
Electric ray (*Torpedo californica*) liver		<0.1
(b) Teleostei		
Bullhead (*Leptocottus armatus*, Girard) liver (f)		16.8
Northern midshipman (*Porichthys notatus*, Girard) liver		16.7
Lingcod (*Ophiodon elongatus*, Girard) liver		4.2
Pacific halibut (*Hippoglossus stenolepis*, Schmidt) liver (f)		10.4
Arrowtooth halibut (*Atheyesthes stomias*, Jordan and Gilbert)	<1	
Sand sole (*Psettichthys melanostictus*, Girard) liver		4.2
Petrale sole (*Eopsetta jordani*, Lockington) liver		<0.1
Dover sole (*Microstomus pacificus*, Lockington) liver (f)		<0.1
English sole (*Parophrys ventulus*, Girard) liver (f)	<1	
Starry flounder (*Platichthys stellatus*, Pallus) liver (f)		<0.1
Perch (*Phanerodon furcatus*, Girard) liver (f)		<0.1
Pacific mackeral (*Pneumatophorus diego*, Ayres) liver		<0.1
Shad (*Alosa sapidissima*, Wilson) liver (f)	2.7	
Rat fish (*Hydrolagus colliei*, Lay and Bennett) liver (f)	<1	
Sable fish (*Anoplopoma fimbria*, Pallus) liver (f)		<0.1
Sable fish (*Anoplopoma fimbria*, Pallus) liver	<1	
Sable fish (*Anoplopoma fimbria*, Pallus) intestine	<1	
Sable fish (*Anoplopoma fimbria*, Pallus) intestinal contents	<1	
Sable fish (*Anoplopoma fimbria*, Pallus) muscle	<1	
Sable fish (*Anoplopoma fimbria*, Pallus) kidney	<1	
Sable fish (*Anoplopoma fimbria*, Pallus) testis	<1	
Sable fish (*Anoplopoma fimbria*, Pallus) gill	<1	
Anadromous fish taken from fresh water		
(a) Fingerling before reaching salt water		
Salmon (*Oncorhynchus tshawytascha*, Walbaum)	<1	
Steelhead (*Salmo gairdneri*, Richardson)	3.5	
(b) Adults prior to spawning		
Salmon (*Oncorhynchus tshawytascha*, Walbaum)	<1	
(c) Adults reared in fresh water		
Striped bass (*Roccus saxatilis* Walbaum) liver	11.5	
Striped bass (*Roccus saxatilis* Walbaum) liver (f)	<1	
Striped bass (*Roccus saxatilis* Walbaum) muscle	<1	
Striped bass (*Roccus saxatilis* Walbaum) intestine	<1	
Striped bass (*Roccus saxatilis* Walbaum) intestinal contents	<1	
Striped bass (*Roccus saxatilis* Walbaum) gill	<1	
Striped bass (*Roccus saxatilis* Walbaum) testis	<1	

TABLE II

(Continued)

Source of enzyme	TMAO synthesized (μmoles/hr/g of tissue)	
	A	B
Fresh water fishes		
Trout (*Salmo gairdneri*, Richardson)		<0.2
Sculpin (*Cottus*)		<0.1
Green sunfish (*Lepomis cyanellus*) liver		2.5
Bluegill (*Lepomis macrochirus*) liver		1.3
Carp (*Cyprinus carpio*) liver	<1	
Carp (*Cyprinus carpio*) kidney	<1	
Channel catfish (*Ictalurus punctatus*) liver	<1	
Channel catfish (*Ictalurus punctatus*) kidney	<1	
Channel catfish (*Ictalurus punctatus*) intestine	<1	
Channel catfish (*Ictalurus punctatus*) gill	<1	
Channel catfish (*Ictalurus punctatus*) muscle	<1	

* Assay reaction mixture contained: A, 0.207 μmole [^{14}C]TMA·HCl of specific activity 207 μC/mmole; B, 1.97 μmole [^{14}C]TMA·HCl of specific activity 21.7 μC/mmole.

** One specimen when assayed by the method of BAKER AND CHAYKIN[4] gave 3 times the activity of hog liver (Table I).

TABLE III

OTHER ANIMALS AND PLANTS* HAVING NO DEMONSTRABLE TMAO SYNTHETIC ABILITY *in vitro*

Microorganisms and animals	
1 *Escherichia coli*	9 Planaria
2 *Azotobacter agilis*	10 Octopus**
3 *Lactobacillus acidophilus*	11 Lumbricus
4 *Clostridium butyricum*	12 *Artemia salina* (Brine shrimp)
5 Euglena	13 Daphnia
6 Amoeba	14 *Cancer magister*** (crab)
7 Paramecia	15 *Pacifastacus leniusculus*** (fresh-water cray fish)
8 Hydra	16 Tenebrio (beetles and larvae)
Plants	
17 Spirogyra	26 *Oryza sativa* L., var. Caloro (rice)
18 Nitella	27 *Lupinus albus* L. (lupine)
19 *Riccia fluitans* L.	28 *Medicago sativa* L., var. Caliverde (alfalfa)
20 *Salvinia rotundifolia* Willd.	29 *Trifolium pratense* L., var. Medium (red clover)
11 *Ceratopteris thalictroides*, Brongn.	30 *Trifolium repens* L., var. Ladino (white clover)
22 Utricularia	31 *Triticum vulgare*, Vill. var. Romona (wheat)
23 *Elodea densa* (Planch.) Casp.	32 *Linum usitatissmum* L., var. Imperial (flax)
24 *Hordeum vulgare* L., var. Cal. Mariout (barley)	33 *Zea Mays* L. (corn)
25 *Pisum sativum* L., var. Alaska (pea)	34 *Solanum tuberosum* L.*** (potato)
Others	
35 Mixed zooplankton and phytoplankton§	

* The entire organism was assayed in all cases not labelled (**) or (***). Each assay reaction mixture contained 0.207 μmole [^{14}C]TMA·HCl of specific activity 207 μC/mmole.

** Only the liver assayed.

*** Only shoots assayed.

§ Taken from the Pacific Ocean in the vicinity of Point Reyes.

water. From the results of feeding experiments with salmon, BENOIT AND NORRIS[9] have shown that this increase can result from retention of TMAO obtained from the diet. In support of this view, we have found that neither the livers of fingerling nor adult salmon oxidize TMA. Of the other teleosts investigated, an apparently unexplainable variation in activity is evident. For example, of the livers from the group of seven phylogenetically similar species of flat fish taken from the same area of the Pacific, two were active and five inactive. For reasons given above, some species showing no activity may under other conditions synthesize TMAO.

Usually only livers have been assayed, but when a number of tissues were assayed in a fish (striped bass) having liver TMAO-synthetic capacity, no other tissue was active. In fish with inactive livers (sable fish and channel catfish), other organs were inactive also. TMAO synthesis by gut flora is unlikely as in two cases (sable fish and striped bass) absence of activity was demonstrated.

TMAO synthesis could not be demonstrated in any invertebrate (Table III). Even zooplankton which contain large quantities of TMAO did not catalyze the oxidation of TMA. This fact increases the likelihood that the enzyme, the distribution of which we have investigated, is one concerned with the detoxidation of TMA (as exemplified particularly by mammalian liver), but in species where TMAO has another role (as an end product of nitrogen metabolism[3], methyl donor[10] or in osmoregulation) an alternate synthetic pathway may be important.

The lack of any demonstrable TMAO synthesis by plants *in vitro* (Table III) made it seem advisable to look for TMAO biosynthesis in plants *in vivo*. Sorghum (*Sorghum vulgare* Pers.) and alfalfa seedlings were fed 1.55 μmoles of TMA·HCl and wheat seedlings were fed 2.07 μmoles of TMA·HCl having a specific activity of 207 μC/mmole. They gave 3.2 %, 12 % and 3.5 % conversions, respectively, to material indistinguishable from TMAO in the assay system. Sufficient radioactivity was contained in the alfalfa extract such that after paper chromatography and treatment of the chromatogram with NH_3 vapors for 2.5 h to remove volatile amines, a radioactive spot was detectable having an R_F of 0.53, identical with that of TMAO. These experiments are of a very preliminary nature and the data are interpreted as being only suggestive of the ability of plants to convert TMA to TMAO.

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