

The Biosynthesis of Trimethylamine-*N*-Oxide*

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Norris and Benoit (1) provided good evidence that injected trimethylamine is converted in the rat to its *N*-oxide and excreted in the urine. Other mammals have been shown to have the ability to effect the same reaction (2, 3).

In a preliminary communication (4), we have reported that hog liver microsomes will catalyze the oxidation of trimethylamine *in vitro*. The additional requirement for reduced triphosphopyridine nucleotide and oxygen makes this system analogous to a group of microsomal enzyme systems that also catalyze biological oxidations (5). Thus, a study of the oxidation of trimethylamine may yield information on microsomal oxidations in general.

In this paper, evidence is presented that the product of trimethylamine oxidation is trimethylamine-*N*-oxide. Results of experiments to determine optimal conditions for this reaction and the effects of some potential inhibitors and activators are reported.

EXPERIMENTAL PROCEDURE

Materials—Unlabeled TMA·HCl was prepared according to the method of Adams and Marvel (6).¹ The product, after recrystallization from *n*-butanol, had a melting point range of 268–272°. Tests for the presence of primary and secondary amines and the ammonium ion were negative. Paper chromatography of the product in *n*-butanol saturated with 25% acetic acid (7) and *n*-butanol-formic acid-water (77:10:13 by volume) was employed to test its homogeneity. Single blue spots with R_F values of 0.59 and 0.26, respectively, were located by a spray reagent containing 0.2% bromocresol green in ethanol. To obtain a uniformly yellow background, acid-washed papers were preferred.

TMA·HCl-¹⁴C was synthesized in 56% yield from formaldehyde-¹⁴C (1.0 mc per mmole) by the method of Sommelet and Ferrand (8). Its chromatographic homogeneity was similarly ascertained.

TMAO was readily prepared by treatment of TMA with 3% hydrogen peroxide (9) and the product (melting point, 98°) was recrystallized from methanol. It was chromatographically homogeneous in *n*-butanol-25% acetic acid (R_F 0.67) and *n*-butanol-formic acid-water (R_F 0.41).

Potassium pyrophosphate was prepared by passage of the sodium salt through a Dowex 50-H⁺ column and titration of the eluate with aqueous potassium hydroxide.

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¹ The abbreviations used are: TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide.

The following chemicals were purchased: sodium *p*-chloromercuribenzoate, Nutritional Biochemicals Corporation; aminopterin, California Corporation for Biochemical Research; gold chloride, Fisher Chemical Company; α, α' -dipyridyl and neocuproine, G. Frederick Smith Chemical Company; TPN, TPNH, glucose-6-P, and glucose-6-P dehydrogenase, Type V, Sigma Chemical Company.

Preparation of Hog Liver Microsomal Fraction—Hog liver, 100 g, was homogenized in a Waring Blendor for 20 seconds with 0.05 M potassium pyrophosphate buffer, pH 8.2, 250 ml. After filtration through cheesecloth to remove cell debris, the mitochondrial fraction was sedimented by centrifugation at 5000 $\times g$ for 10 minutes. Separation of the microsomal fraction from supernatant proteins was most conveniently achieved by employing the ammonium sulfate precipitation method of Strittmatter and Velick (10). All operations were carried out at 4°, and the microsomes were finally stored as a suspension in pyrophosphate buffer at -12°. In this state, microsomal activity was retained indefinitely. In preparation for the determination of protein by a biuret procedure (11), microsomes were dispersed by the addition of sodium deoxycholate to a concentration of 5%.

Assay for Biosynthesized TMAO-¹⁴C—After incubation, reaction mixtures, 2 ml, were deproteinized with 50% perchloric acid, 0.10 ml, and centrifuged. Aliquots, 1.0 ml of the supernatant solution were applied to columns containing Dowex 50-H⁺, 500 mg. Nonexchangeable ions were washed from the column by water, 4 ml. TMAO-¹⁴C, which is retained by the resin, was eluted with 5% aqueous ammonia, 2 ml. Aliquots, 0.5 ml, were plated on stainless steel planchets and evaporated to dryness under an infrared lamp before counting their radioactivity at infinite thinness in a gas flow counter.

With this assay system, it was found that the TMAO incubated with hog liver homogenate or microsomes underwent no significant reduction. No other reaction products were identifiable on a chromatogram of the reaction mixture. Therefore, the oxidation of TMA may be studied even in crude preparations without concern for further metabolism.

Optimal Reaction Conditions—The amount of TMAO formed as a function of time of incubation is plotted in Fig. 1. A linear rate of TMAO synthesis is evidenced during the first 20 minutes; so this time of incubation was chosen for subsequent experiments. The composition of the reaction mixture is given below this illustration.

The effects on TMAO synthesis of varying TPN, glucose-6-P, TMA·HCl, and microsome concentrations, and pH are plotted in Figs. 2 to 4. The concentrations or conditions chosen for routine use are indicated in the figures by *arrows*.

All reaction mixtures contained an excess of glucose-6-P dehydrogenase. It was shown that a 10-fold increase in glucose-

6-P dehydrogenase concentration caused no increase in the rate of TMAO biosynthesis.

Preparation of Biosynthesized TMAO Hydrochloride—A mixture containing: hog liver microsomes, 100 mg of protein; TPN, 30 mg; glucose-6-P, 110 mg; excess glucose-6-P dehydrogenase; TMA·HCl-C¹⁴, 28 mg, 1.19×10^6 d.p.m.; and 0.05 M potassium pyrophosphate buffer, pH 8.2, 50 ml; were incubated at 37° for 3 hours, and 50% perchloric acid was added to a final concentration of 2.5%. Dowex 1-HCO₃⁻ was stirred in until the mixture was neutral to pH paper. After centrifugation, the supernatant solution was decanted and lyophilized. The lyophilized powder was extracted with ethanol to remove most of the remaining inorganic salts, and the extract was evaporated to dryness at 40° under reduced pressure. Dissolved in a small

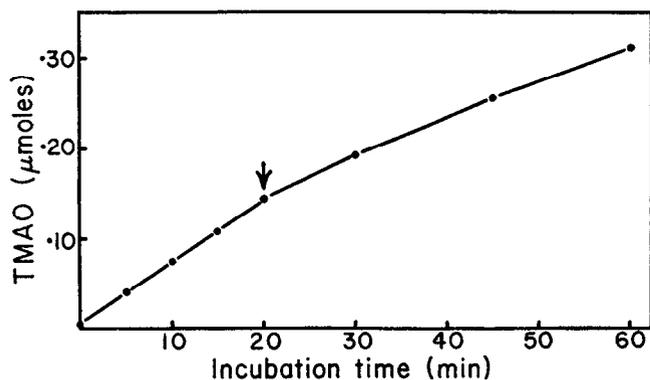


FIG. 1. Progress curve. The reaction mixture contained: hog liver microsomes, 2.0 mg, in 0.05 M potassium pyrophosphate, pH 8.2; TMA·HCl-C¹⁴ (specific activity, 21.66 μc per mmole), 189 μg; TPN, 1.0 mg; glucose-6-P, 2.0 mg; glucose-6-P dehydrogenase, 0.1 unit (12), and was made up to 2 ml with 0.05 M potassium pyrophosphate, pH 7.7.

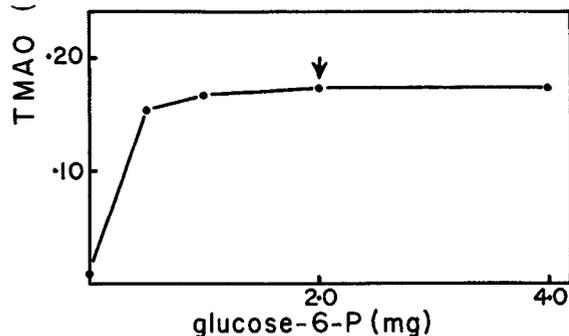
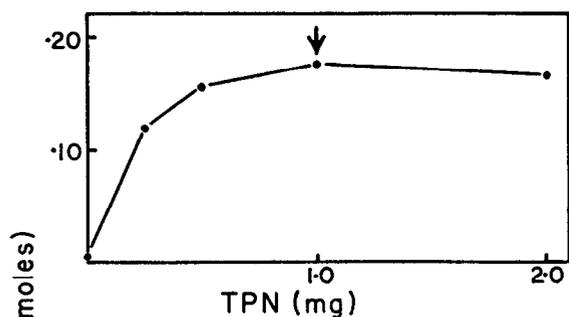


FIG. 2. Effect of TPN and glucose-6-P concentrations on TMAO biosynthesis. Reaction conditions employed to obtain the data plotted in Figs. 2 to 4, except for the variables under study, were the same as given below Fig. 1.

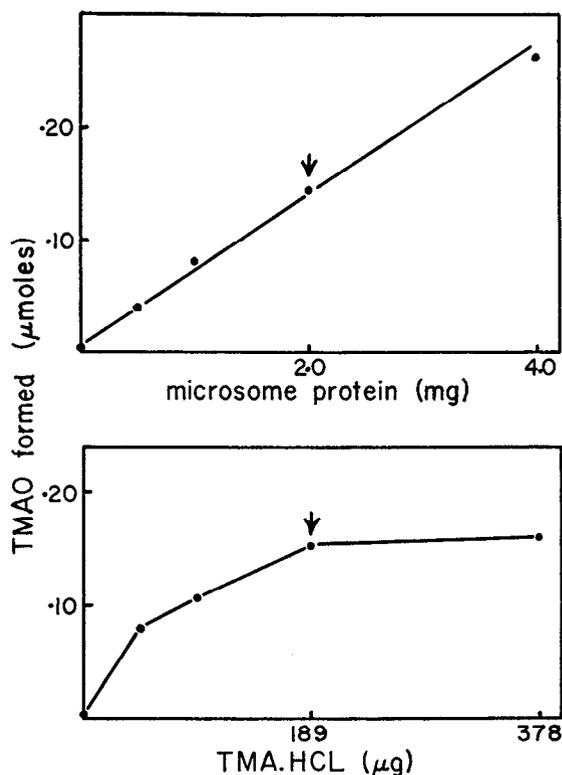


FIG. 3. Effect of microsome and TMA·HCl concentrations on TMAO biosynthesis.

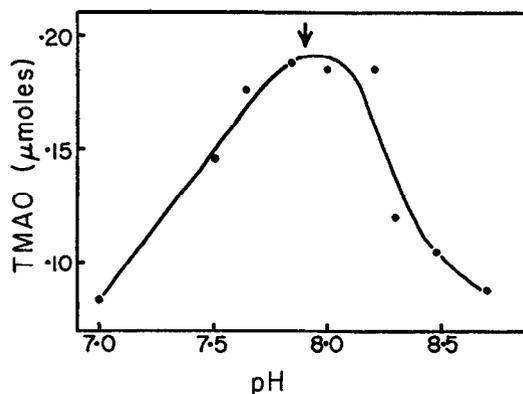


FIG. 4. pH curve

volume of water, it was placed on a column (15 × 2 cm) of Dowex 50 W-X8 (H⁺) (200 to 400 mesh). The column was washed with water before eluting the TMAO-C¹⁴ with 1% aqueous ammonia and collecting 5 ml fractions. Radioactivity was detected in Fractions 119 to 133, which were bulked and evaporated to dryness under reduced pressure at less than 50°.

The residue was taken up in water (0.1 ml), and TMAO aurichloride was precipitated by addition of 30% aqueous gold chloride (0.5 ml). The precipitate was washed twice with small volumes of water before drying it over phosphorus pentoxide under reduced pressure.

To convert to the hydrochloride, the aurichloride was dissolved in 0.1 N hydrochloric acid, placed on a Dowex 50W-X8 (H⁺) column (3 × 0.8 cm), washed well with water, and developed with 2.5 N hydrochloric acid. The product was detected by its

TABLE I

Identity of product of trimethylamine bio-oxidation

Analyses for C, H, N, and Cl, which are given on a percentage of dry weight basis, were made from a 25 to 30 mg sample. Values obtained for C, H, N, and Au agreed with the calculated value within the limits of experimental error for the microanalytical procedures used.

$(\text{CH}_3)_3\text{NO}\cdot\text{HAuCl}_4$	Melting point	Elemental analyses				
		C	H	N	Au	Cl
Biosynthesized sample	256–257°	8.51	2.39	3.29	46.3	33.22
Authentic sample	256–258°	8.77	2.16	3.45	45.4	33.54
Calculated		8.68	2.43	3.38	47.48	34.17

radioactivity; the radioactive fractions were combined and evaporated to dryness at room temperature.

Proof of Identity of Biosynthesized TMAO—The results of elemental analyses and melting point determinations performed upon derivatives of authentic and biosynthesized TMAO are tabulated in Table I. There is good agreement between the two samples for both of these properties.

The infrared spectra of biosynthetic and authentic TMAO hydrochloride are reproduced in Fig. 5. The two samples show identical absorption maxima. The absorption band at approximately 10.5μ is due to N—O stretching (13).

Effect of Some Potential Inhibitors (Table II)—Potassium cyanide, 0.01 M, failed to inhibit TMAO formation; so it was concluded that if any contaminating mitochondrial cytochrome-

dependent electron system was present, it was not involved in this oxidation.

Catalase does not inhibit the synthesis, which suggests that hydrogen peroxide is not the intermediate oxidizing agent. Neither did significant inhibition nor enhancement result from the inclusion in the reaction mixture of aminopterin, sodium chloromercuribenzoate, EDTA, or the specific chelators of iron (Fe^{++} and Fe^{+++}) and copper (Cu^+), α, α' -dipyridyl, and neocuproine, respectively.

Proof of Source of TMAO Oxygen—To prove that the oxygen atom contained in the product is derived from molecular oxygen, the biosynthetic reaction was performed both in the presence of an O_2^{18} atmosphere and O^{18} -labeled water. The TMAO formed

TABLE II

Effect on TMAO synthesis of some potential inhibitors

The reaction mixture in each case had the same composition as given below Fig. 1.

Inhibitor	Relative TMAO-synthesizing activity	
	Inhibitor concentration	
	M	%
None		100
KCN	1×10^{-2}	108
Catalase (approximately 1 mg)		105
Sodium <i>p</i> -chloromercuribenzoate	5×10^{-5}	89
EDTA	10^{-2}	89
Aminopterin	10^{-3}	96
α, α' -Dipyridyl	10^{-3}	111
Neocuproine	10^{-3}	105

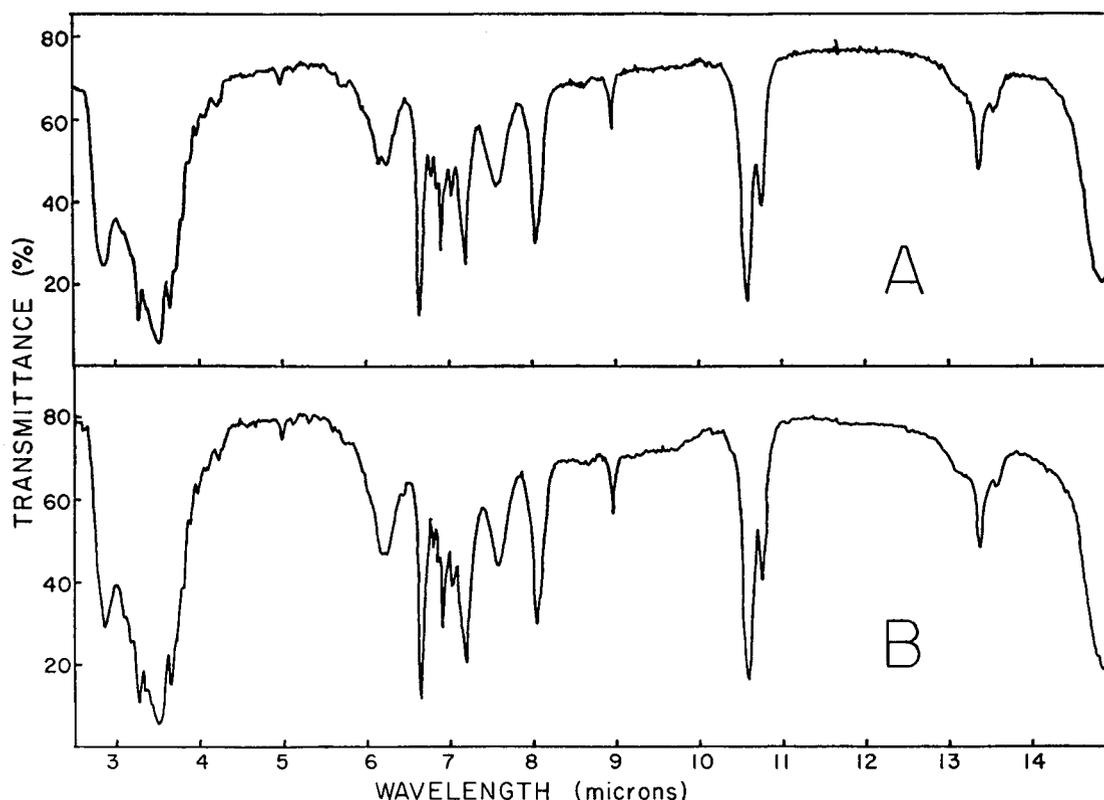


FIG. 5. The infrared spectra of (A) authentic and (B) biosynthesized $(\text{CH}_3)_3\text{NO}\cdot\text{HCl}$. The sample (approximately 1 mg) and KBr (400 mg) were obtained as an intimate mixture by lyophilization from aqueous solution before pressing the pellet.

was isolated by the procedure outlined above. The oxygen moiety of this product was converted to carbon dioxide by heating in an evacuated sealed tube with mercuric chloride at 400° (14).

O¹⁸ enrichment was evident only in duplicate samples derived from the reaction mixture incubated under O¹⁸ oxygen (Table III).

TMAO is extremely deliquescent, and probably the degree of enrichment does not approach the theoretical level because of water present in the product analyzed. Nonetheless, the experiment clearly shows that the atmosphere is the source of the oxygen in the product.

TPNH Uptake—TMA·HCl-C¹⁴ was incubated with hog liver microsomes for 20 minutes in the presence of TPNH (Table IV). Assays for TPNH consumed and TMAO-C¹⁴ formed were made immediately after incubation. For the former, the fluorimetric

TABLE III

Source of TMAO oxygen

Line *a*: The reaction mixture contained: TMA·HCl-C¹⁴, 15 mg; TPN, 27 mg; glucose-6-P, 100 mg; hog liver microsomes, 100 mg; excess glucose-6-P dehydrogenase; and was made up to 50 ml with 0.10 M sodium pyrophosphate, pH 8.2. Incubation under O₂¹⁸ (concentration given on Line *c*) was at 37° for 3½ hours. Isolation of TMAO·HCl, according to the scheme given above, gave 7.0 mg of product. This was diluted with an equal weight of authentic TMAO for O¹⁸ assay. Line *b*: The reaction mixture (50 ml) contained 25 ml of H₂O¹⁸ (concentration given on Line *d*). The other reaction constituents were as above. During incubation, the vessel was open to the atmosphere. TMAO·HCl, 4.1 mg, was isolated and diluted with an equal weight of authentic TMAO before O¹⁸ assay.

	O ¹⁸ found	Excess O ¹⁸
	atom % excess	theoretical atom %
<i>a</i> . TMAO from incubation with O ₂ ¹⁸	0.259	0.758
<i>b</i> . TMAO from incubation with H ₂ O ¹⁸	0.000	0.401
<i>c</i> . O ₂ ¹⁸	1.515	
<i>d</i> . H ₂ O ¹⁸	1.605	

TABLE IV

TPNH consumption during TMAO biosynthesis

The complete reaction mixture contained: hog liver microsomes, 1.0 mg; TPNH, 0.10 μmole; and TMA·HCl-C¹⁴, 189 μg; and was made up to 2 ml with 0.05 M potassium pyrophosphate, pH 7.7. Incubation was at 37° for 20 minutes.

	<i>a</i> . TPNH	<i>b</i> . TMAO formed
	μmoles	
1. Complete reaction mixture, incubated.....	15.1	26.4
2. Reaction mixture minus TMA·HCl.....	31.2	
3. Complete reaction mixture, not incubated..	79.2	8.2
TPNH consumed during TMAO synthesis (Line 2 <i>a</i> minus Line 1 <i>a</i>).....	16.1	
TMAO formed during incubation (Line 1 <i>b</i> minus Line 3 <i>b</i>).....		18.2
Molar ratio of TPNH consumed to TMAO formed.....	0.88:1.00	

TABLE V

Oxygen consumption during TMAO biosynthesis

The complete reaction mixture contained: hog liver microsomes, 10.8 mg; TPN, 1.8 mg; glucose-6-P, 3.6 mg; TMA·HCl-C¹⁴, 340 μg; and excess glucose-6-P dehydrogenase, and was made up to 3.6 ml with 0.05 M potassium pyrophosphate, pH 8.2. Incubation was at 37° for 3 hours.

	O ₂ consumed	TMAO formed
	μmoles	
1. Complete reaction mixture.....	6.17	1.82
2. Reaction mixture minus TMA·HCl.....	4.60	
O ₂ consumed during TMAO biosynthesis, (Line 1 minus Line 2).....	1.57	
Molar ratio of O ₂ consumed to TMAO formed...	0.86:1.00	

method of Lowry *et al.* (15) with some modifications introduced by Bassham *et al.* (16) was employed.

Considerable loss of TPNH occurs during incubation in the absence of substrate, for which oxidation to TPN is largely responsible. Neither the incorporation of nicotinamide (0.02 M) nor cyanide (0.02 M) in the reaction mixture reduces this loss. The amount of TPNH consumed and attributable to TMAO biosynthesis was calculated and compared on a molar basis with TMAO formed. The ratio is 0.88:1.00, respectively.

Oxygen Consumption—TMA·HCl-C¹⁴ was incubated with hog liver microsomes and a TPNH-generating system. The oxygen consumed was determined in a Warburg apparatus (Table V). Results with controls indicated that in the absence of substrate considerable uptake of oxygen still occurred.

TMA-dependent oxygen uptake was calculated by correction for substrate-independent oxygen consumption. A determination was made of the amount of TMAO synthesized in each flask. The molar ratio of oxygen consumed to TMAO formed is 0.86:1.00.

DISCUSSION

Choline, when present in the food, has been reported by Norris and Benoit (1) and by Lintzel (2) to be largely converted to TMA and TMAO before excretion in the urine. Although the breakdown to TMA has been attributed to intestinal bacteria, our work shows the availability of an endogenous liver enzyme that may detoxify TMA by oxidation to TMAO. The extent of distribution of this enzyme with regard to different tissues and species, and its biological significance, are being determined and will be reported elsewhere.

The determination of optimal conditions for this biosynthetic reaction *in vitro* is a necessary prerequisite for later study of fractionation procedures aimed at the extraction of the microsomal enzyme. Many microsomal enzymes have resisted exhaustive efforts to extract them, but the recent success of Imai and Sato (17) in obtaining soluble aniline hydroxylase, and Isselbacher (18) with a glucuronyl transferase from microsomes indicates some possibility of success. Both workers used a heat-treated venom from *Trimeresurus flavoviridis*.

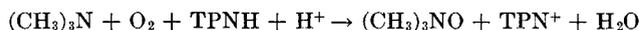
Since a large number of substrates are hydroxylated by microsomes with similar cofactor requirements, the question arises, "Is one nonspecific enzyme responsible?" Posner *et al.* (19)

have some evidence, based on the ability of certain inhibitors to differentially affect the hydroxylation of various substrates, that many enzymes are involved. Another possibility is that for each hydroxylation a complex of enzymes is required, any member of which may be common to other hydroxylating complexes. In view of these uncertainties, it is premature to name the system that oxidizes TMA.

In our own studies, EDTA and specific chelators of iron and Cu^+ gave partial inhibition or enhancement. Since this is an unfractionated microsomal enzyme system, it is not possible to draw conclusions concerning possible metal requirements for TMAO synthesis. Danger lies in extraneous factors masking the effect under study. As aminopterin does not inhibit, our system is probably unrelated to the phenylalanine hydroxylase of Kaufman (20), which requires a folic acid-like cofactor for activity.

The suggestion of Müller and Immendorfer (3) that the mechanism of TMAO biosynthesis in mammalian liver involves a hydration and a dehydrogenation of TMA led us to check the source of TMAO oxygen. Molecular O_2^{18} , but not the O^{18} of H_2O^{18} , was incorporated; therefore, a hydration mechanism is not tenable.

The consumption of TPNH and oxygen concurrent with TMAO formation has been determined and permits the formulation of the reaction as follows:

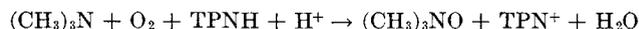


SUMMARY

An assay has been developed that permits rapid counting of biosynthesized trimethylamine-*N*-oxide- C^{14} at infinite thinness. With this method, the properties of a cell-free system from hog liver, which catalyzes the oxidation of trimethylamine to trimethylamine-*N*-oxide, have been investigated.

A procedure is given for isolating the biosynthesized trimethylamine-*N*-oxide from the reaction mixture, and the properties of two derivatives have been compared with authentic samples.

The stoichiometry of the reaction is:



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