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TRIMETHYLAMINE N-OXYGENATION AND N-DEMETHYLATION IN RAT LIVER MICROSOMES

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Abstract—The *in vitro* oxidation of trimethylamine (TMA) to TMA *N*-oxide (TMAO) and dimethylamine (DMA) was studied in rat liver microsomes. Pretreatment of rats with phenobarbital, 3-methylcholanthrene, ethanol or pregnenolone 16α-carbonitrile had little or no effect on the liver microsomal metabolism of TMA to TMAO or DMA. Changing the atmosphere in the incubation vessel from 20% oxygen/80% nitrogen (air) to 100% oxygen had a selective stimulatory effect on the N-oxygenation of TMA but did not affect TMA N-demethylation. In addition, the K_m for TMA N-demethylation was 5-fold higher than for the N-oxygenation reaction. The results of these studies suggest that the enzyme systems responsible for N-demethylation and N-oxygenation are different and that they are under different regulatory control. Carbon monoxide (CO/O_2 = 80/20) had little or no inhibitory effect on either the N-demethylation or N-oxygenation of TMA by liver chromosomes from control or pregnenolone 16α-carbonitrile-treated rats. Additional studies indicated that methimazole, an inhibitor of FAD-containing monooxygenase (FMO), was a potent inhibitor of TMA oxidation. Preincubation of liver microsomes from control or pregnenolone 16α-carbonitrile-treated rats at 37° for 10 min without NADP(H) (a procedure that irreversibly inactivated FMO activity) resulted in >95% inhibition of TMA N-demethylation and N-oxygenation, and this inhibition was prevented by including a NADPH-generating system in the preincubation medium (a procedure for preventing the thermal inactivation of FMO activity). The data suggest that FMOs are the major enzymes responsible for N-demethylation and N-oxygenation of TMA in rat liver microsomes.

Trimethylamine (TMA§) is formed from dietary choline by colon bacteria and by reduction of dietary TMA *N*-oxide (TMAO), which is present in high levels in fish. In humans, TMA is oxidized to TMAO (major metabolite) and dimethylamine (DMA, minor metabolite), which are excreted in the urine along with small amounts of unmetabolized TMA [1, 2]. In these studies, about 95% of an administered dose of TMA is oxidized and excreted in the urine as TMAO, whereas less than 1% of a dose of TMA is demethylated and excreted in the urine as DMA [1]. Trimethylaminuria is the result of a heritable metabolic defect in the oxidation of TMA to TMAO [2–4]. Since TMA smells like rotten fish, individuals who suffer from this disease experience poor social interactions and have even had suicidal tendencies [3–7].

Although earlier studies indicated that many tertiary amines are oxidatively metabolized by a purified flavin-containing monooxygenase (FMO) from liver microsomes [8–11], relatively few detailed studies on the metabolism of TMA or on factors that influence TMA metabolism have been reported. Studies with purified mouse and pig liver FMO revealed TMA-stimulated oxygen consumption, TMA-stimulated NADPH oxidation and TMA-dependent formaldehyde (HCHO) formation, indicating the presence of TMA-oxidizing activity in these enzyme preparations [9]. Although similar results were obtained with mouse and pig liver microsomes [12], the K_m for TMA metabolism by the purified FMO was more than 10-fold higher than the K_m obtained from studies with mouse and pig liver microsomes [9, 12]. The first demonstration of the enzymatic N-oxygenation of TMA was reported by Goldstein *et al.* [13, 14]. In this study, metabolism of [^{14}C]TMA to [^{14}C]TMAO by shark liver slices was observed. Although purified mammalian liver FMO was found to metabolize TMA [11], a yeast microsomal cytochrome P450 has been reported to oxidize TMA, which has also been indicated elsewhere as a P450 substrate [15], but it may be relevant only to yeast. Various bacteria contain a cytosolic TMA dehydrogenase (EC 1.5.99.7), which is coupled with electron-transporting flavoproteins and which oxidizes various tertiary amines [16]. This enzyme system is neither cytochrome P450 nor FMO.

The present study was initiated to evaluate the role of flavin-containing monooxygenase and cytochrome P450 in the N-oxidation and N-demethylation of TMA in rat liver microsomes and to investigate the effects of microsomal enzyme inducers on the metabolism of TMA. If any inducible cytochrome P450 is involved in TMA oxidation, its stimulation may offer a potential treatment of the human trimethylaminuria syndrome.

MATERIALS AND METHODS

Chemicals. TMS·HCl, DMA·HCl, mono-

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§ Abbreviations: TMA, trimethylamine; DMA, dimethylamine; MMA, monomethylamine; TMAO, TMA *N*-oxide; and FMO, FAD-containing monooxygenase.

methylamine (MMA)·HCl and TMAO·$2H_2O$ were obtained from Eastman Kodak (Rochester, NY); sodium phenobarbital, pregnenolone 16α-carbonitrile, 3-methylcholanthrene, glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase were from the Sigma Chemical Co. (St. Louis, MO); and ethanol, K_2CO_3, KOH, Na_2WO_4, $ZnSO_4$, $Ba(OH)_2$, HCl, trichloroacetic acid and $TiCl_3$, 20% solution, were from Fisher Scientific (Springfield), NJ.

Animals and treatments. Male Sprague–Dawley rats, 180–200 g, were fed laboratory rat chow and water *ad lib.* for 1 week after receipt from the Charles River Co. To induce different forms of cytochrome P450, the rats were subjected to the following treatments: (a) ethanol, administered as a 15% water solution for 3 days, rats killed on day 4; (b) sodium phenobarbital, i.p. in saline, 75 mg/kg/day for 3 days, rats killed on day 4; (c) 3-methylcholanthrene, i.p. in corn oil, 25 mg/kg/day for 3 days, rats killed on day 4; (d) pregnenolone 16α-carbonitrile, i.g. in corn oil at five doses, 25 mg/kg each, twice daily, killed 12 hr after dose 5; and (e) control rats were untreated or given a single i.g./i.p. dose of corn oil, 0.5 mL/rat 24 hr before being killed.

Liver microsomes. Thirty-three per cent liver homogenates (in 154 mM KCl/50 mM Tris, pH 7.4, buffer) were prepared in a Potter–Elvehjem glass/teflon homogenizer and centrifuged at 9,000 *g* for 20 min at 4°. The supernatant fractions were then centrifuged at 105,000 *g* for 60 min washed once with a solution containing 154 mM KCl and 10 mM EDTA, resuspended in 0.25 M sucrose (15–20 mg protein/mL), and stored at −70°.

Enzyme assays. The incubation mixtures contained, in a 2- or 2.5-mL volume, final levels of 154 mM KCl, 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 0.4 mM NADP, 10 mM glucose-6-phosphate, 0.5 IU/mL, glucose-6-phosphate dehydrogenase, 1 or 1.5 mg/mL microsomal protein, and appropriate concentrations of TMA·HCl in 0.01 M HCl. The initial TMA concentration used in TMA disappearance studies was 40 or 80 μM, and a 250-μM TMA concentration was used for studies on the formation of HCHO, DMA or TMAO to provide near-maximum velocity. Even at 1250 μM, TMA was not inhibitive. The apparent K_m for the formation of DMA and TMAO was estimated using an initial TMA concentration of 5, 10, 20, 50, 100, 250 and 500 μM. Incubations were for 10 min at 37°.

Unless specified otherwise, the components (except TMA) were mixed on ice in 22-mL Perkin–Elmer head space vials, and warmed for 2 min at 37° with shaking in a water bath; each vial was capped with a teflon-lined butyl rubber septum, and TMA·HCl in 0.01 M HCl was injected (1/10 of final volume) through the septum to start the enzyme reaction. After 10 min, incubations aimed at HCHO or TMAO determination were stopped by cooling the vials on ice and immediately injecting 25% $ZnSO_4$ (1/10 of incubation volume) and vortexing the samples. Then a saturated solution of $Ba(OH)_2$ (1/10 of incubation volume) was added, and the samples were again vortexed. The protein precipitates were centrifuged and the clear supernatants used.

For analysis of TMA and DMA, the reaction was terminated by chilling on ice, injecting 1/4 volume of 3 M HCl through the septum, and vortexing. Then 12% Na_2WO_4 (3/20 of incubation volume) was injected and the sample was vortexed again.

Sample processing and analysis. For the gas chromatographic/head space analysis of TMA, DMA and MMA, the reported method [6, 17] was modified. An aliquot of the acidic supernatant was transferred into a clean Perkin–Elmer gas chromatography-head space vial, frozen at −20°, covered with 1.5 mL of 0.01 M HCl, and refrozen. This overlay prevented amine loss in the next step. Just before gas chromatography-head space analysis, 4 g of dry K_2CO_3 and 1 mL of 80% KOH solution were quickly added. The vials were immediately capped with teflon-lines septa and kept in a boiling (100°) water bath for 30 min; then 2 mL of the head space were injected into a Perkin–Elmer gas chromatograph with a gas-tight Hamilton syringe "washed" by aspirating about 0.2 mL of 80% KOH solution and flushing the syringe several times with air to drive out excess KOH. This wash prevented amine binding to the metal needle, which would otherwise diminish the TMA peak and, at lower concentrations, completely bind DMA and MMA.

For TMAO analysis, an aliquot of the clear $ZnSO_4/Ba(OH)_2$ supernatant of the sample (2 mL) was made alkaline with 2 mL of 0.5 M NaOH and evaporated to dryness at 80–85° under vacuum on a rotary evaporator. The dry evaporate was dissolved in 2 mL of 1 M HCl, flushed with nitrogen, and frozen. For reduction of TMAO to TMA, a 20% $TiCl_3$ solution was added to the frozen TMAO in HCl, to obtain a 5% $TiCl_3$ solution. The vial was flushed with N_2 and capped with teflon-lined septum, and the sample was incubated at 25° for 60 min with mild shaking (30 oscillations/min). The sample was then immediately frozen at −50°, and the frozen sample was covered with 1.5 mL of 0.01 M HCl and refrozen. The analysis of amines by the gas chromatography head space (K_2CO_3, KOH, capping) was similar to that described for TMA, DMA and MMA (see above). In these studies, we omitted the use of HCHO [18] in the reducing mixture because TMAO was reduced sufficiently using only $TiCl_3$ solution and HCl. Moreover, HCHO formed condensation products detected by gas chromatography with the FID detector, which were apparently responsible for a modification of the column filling after injecting 100–150 samples, manifested by binding of the amines and tailing of the amine peaks. Furthermore, it required the use of a thermal gradient (65–170°) and about 20 min for the analysis of one sample. By omitting HCHO, the analysis of one sample took only 5 min and at least 500 samples could be analyzed without the apparent difficulties mentioned above. It also became obvious that with the massive amounts of HCHO in the sample the NPD (nitrogen-phosphorus) detector did not respond to low TMA levels that were already noticeable by its typical smell as TMA was leaving the gas chromatograph.

Gas chromatographic analysis. The gas chromatography-head space analysis was made on a Perkin–Elmer 8500 Series gas chromatograph with an NPD

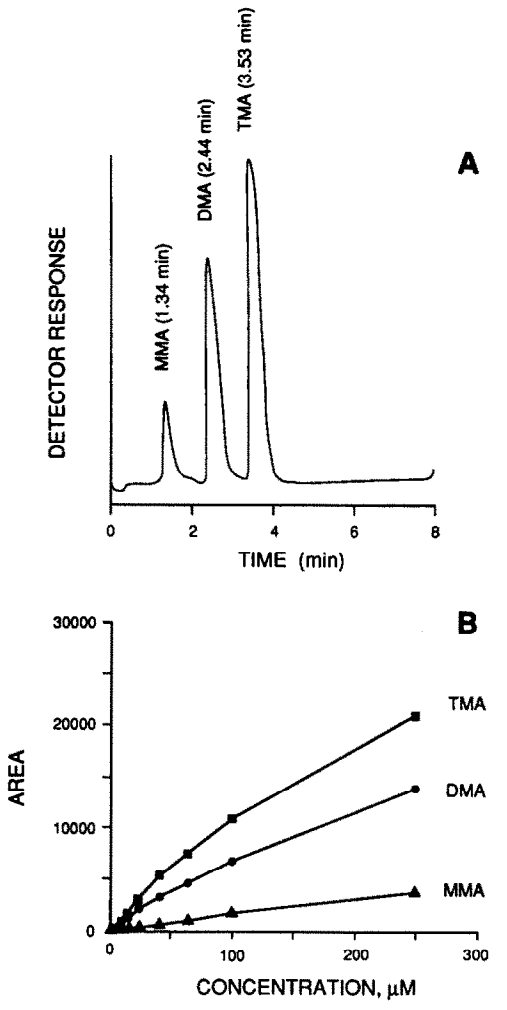

Fig. 1. Gas chromatography-head space analysis of trimethylamine (TMA), dimethylamine (DMA) and monomethylamine (MMA). (A) A typical gas chromatogram. (B) Typical calibration curves after adding the indicated concentrations of amines to incubation vessels.

detector. The head space gas was injected into a glass/lined injection port. The glass column (2 m, 2 mm i.d.) was packed with Carbopack B/4% Carbowax 20 M/0.8% KOH (Suppelco, Belafonte, PA). Amines were eluted with hydrogen (15 mL/min). The temperature of the injection port was 250°, the detector was held at 300°, the column temperature was 65°, and the sample analysis time was 5 min. Peak separations (Fig. 1A) and calibration curves (Fig. 1B) were favorable. The elution times were about 1.3 min for MMA, 2.4 min for DMA, and 3.5 min for TMA. The use of *n*-propylamine or isopropylamine as an internal standard was not satisfactory since the peak areas were more variable than with TMA and dependent on sample composition (protein precipitation chemicals, sample volume, and head space temperature). TMA, DMA, MMA and TMAO standards were used for calculation of the results. The peak areas were calculated by a built-in integrator.

RESULTS

Kinetic experiments. Initial experiments showed that the rate of TMA oxidation was constant for at least 10 min, and the amounts of NADP (0.4 mM), glucose-6-phosphate (10 mM) and glucose-6-phosphate dehydrogenase (0.5 IU/mL) added to the incubation mixture were optimal (data not shown).

The apparent K_m values for the metabolism of TMA and DMA and TMAO were determined. The K_m value for DMA formation in five independent experiments with control microsomes was 92 ± 16 μM (mean ± SD) and the K_m for TMAO formation was 18.2 ± 5.7 μM (data not presented). These values are theoretical initial concentrations in the incubation medium just after the injection of the TMA·HCl solution. At pH 7.4, TMA·HCl changes immediately to TMA which is volatile, and during the fifth minute of incubation only 7% of the TMA was in the 2-mL water (microsomal) phase, and 93% was in the 20-mL gas phase of the incubation vials (partition coefficient was calculated to be 0.86). Since the partition coefficient is independent of concentration, the K_m values could be recalculated to give a K_m of 7.33 ± 1.33 μM for DMA formation and 1.44 ± 0.45 μM for TMAO formation.

Effects of microsomal enzyme inducers. In rats pretreated with phenobarbital, 3-methylcholanthrene, or ethanol for 3 days, there was little or no effect on the N-demethylation or N-oxygenation of TMA (Fig. 2). In some studies where pregnenolone 16α-carbonitrile was administered once every 12 hr for five doses and the animals killed 12 hr after the fifth dose, there was a small increase in the N-demethylation of TMA but not in the N-oxygenation reaction, whereas in other experiments there was no effect on either the demethylation or N-oxygenation. The overall conclusion of our studies was that pretreatment of rats with phenobarbital, 3-methylcholanthrene, ethanol or pregnenolone 16α-carbonitrile had little or no effect on the N-oxygenation or demethylation of TMA, and there was no evidence that inducible cytochrome P450 catalyze these reactions.

Effects of inhibitors of cytochrome P450 and FMO. Studies were initiated to investigate the effects of carbon monoxide (CO) on the metabolism of TMA by rat liver microsomes. It was found that a CO/O_2 ratio of 1 (50% CO/50% O_2) or 4 (80% CO/20% O_2) did inhibit TMAO or DMA formation by microsomes from control or pregnenolone 16α-carbonitrile-pretreated rats (Fig. 3). A CO/O_2 ratio of 9 (90% CO/10% O_2) had little or no inhibitory effect on TMA metabolism by control microsomes (the demethylation and N-oxygenation combined was unchanged) and a small inhibitory effect (30%) on TMA metabolism by microsomes from pregnenolone 16α-carbonitrile-treated rats. The small inhibitory effect on TMA metabolism that was observed may have resulted from a decrease in O_2 tension since the N-oxygenation of TMA was more rapid in 100% oxygen and even 50% oxygen (despite the presence of 50% CO) than in air. The overall

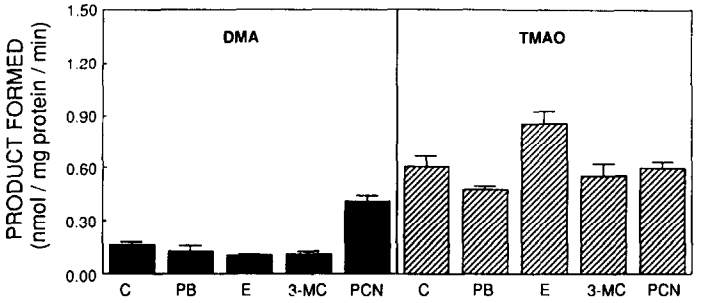

Fig. 2. Effect of pretreatment of rats with inducers of cytochrome P450 on the metabolism of trimethylamine (TMA) by rat liver microsomes. Metabolism of TMA to dimethylamine (DMA) and trimethylamine *N*-oxide (TMAO) by control rat liver microsomes (C) and by liver microsomes from rats treated with phenobarbital (PB), ethanol (E), 3-methylcholanthrene (3-MC) or pregnenolone 16α-carbonitrile (PCN) was determined. Values are means ± SD, N = 3–4.

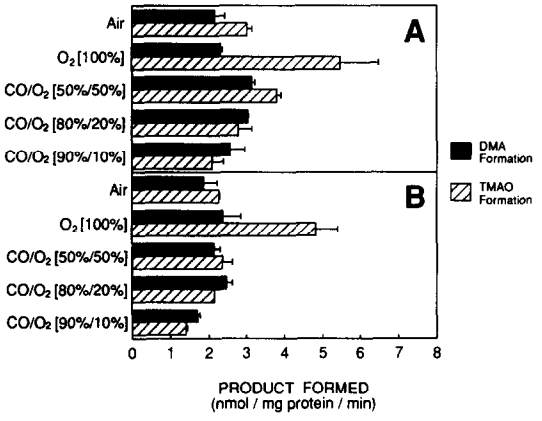

Fig. 3. Effect of carbon monoxide and oxygen concentration on trimethylamine metabolism to dimethylamine (DMA) and trimethylamine *N*-oxide (TMAO) by microsomes from (A) control and (B) pregnenolone 16α-carbonitrile-pretreated rats. Values are means ± SD, N = 3–4.

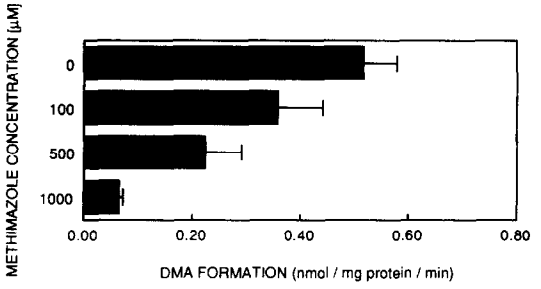

Fig. 4. Effect of methimazole on trimethylamine metabolism to dimethylamine (DMA) by rat liver microsomes. Values are means ± SD, N = 3–4.

results indicate that CO had little or no inhibitory effect on the demethylation and N-oxygenation of TMA. The data also indicate that changing the atmosphere in the incubation vial from 20% oxygen/80% nitrogen (air) to 100% oxygen and even 50% oxygen/50% CO had a selective stimulatory effect on the N-oxygenation of TMA and did not affect TMA demethylation.

The importance of the microsomal flavin-containing monooxygenase for the metabolism of TMA was evaluated with methimazole (a substrate and potent inhibitor of this enzyme) [11]. The results (Fig. 4) indicate that methimazole caused a concentration-dependent inhibition in the metabolism of TMA to DMA, suggesting that TMA demethylation is catalyzed by flavin-containing monooxygenase.

Effect of "thermal inactivation" of flavin monooxygenase. Since flavin monooxygenase is irreversibly inactivated by incubation at 37° for 30 min in the absence of NADPH [11], whereas cytochrome P450 retains catalytic activity under these conditions, we investigated the effect of preincubating liver microsomes without or with the NADPH-generating system at 37° on the subsequent metabolism of TMA in the presence of NADPH. We selected a 10-min preincubation time to decrease the possibility of cytochrome P450 inactivation. The results demonstrated that preincubation of liver microsomes from control or pregnenolone 16α-carbonitrile-pretreated rats for 10 min at 37° in the absence of the NADPH-generating system resulted in more than 95% inhibition of TMA N-oxygenation and N-demethylation (Fig. 5). The addition of an NADPH-generating system during the 10 min/37° preincubation (prevention of thermal inactivation of flavin-containing monooxygenase) protected the TMA-oxidizing activity of liver microsomes.

In additional studies, it was observed that even a 2-min preincubation of liver microsomes at 37° in the absence of NADPH resulted in a 60–80% decrease in the rate of TMA metabolism as measured by TMA disappearance and DMA formation. These results indicate the importance of flavin monooxygenase for TMA N-oxygenation and N-demethylation by rat liver microsomes.

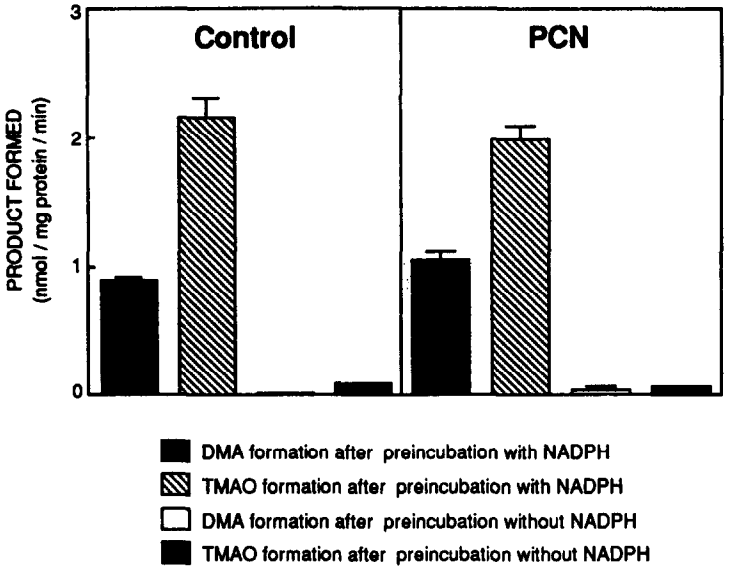

Fig. 5. Thermal inactivation of trimethylamine metabolism. Microsomes from control or pregnenolone 16α-carbonitrile-treated rats were preincubated for 10 min at 37° in the presence or absence of an NADPH-generating system. Trimethylamine metabolism to dimethylamine (DMA) or trimethylamine *N*-oxide (TMAO) was measured. Results are means ± SD, N = 3–4.

DISCUSSION

The results of the present study demonstrated the metabolism of TMA to DMA and TMAO by rat liver microsomes. Evidence was presented that N-oxygenation and N-demethylation of TMA are catalyzed by one or more FMO systems and that cytochrome P450 enzymes do not play a major role in the metabolism of TMA to TMAO or DMA by rat liver microsomes (Figs. 2–5). These conclusions on the importance of flavin-containing monooxygenase for TMA metabolism by rat liver microsomes were based on direct measurements of the formation of TMA metabolites and are in agreement with conclusions from studies on TMA-stimulated NADPH and oxygen consumption utilizing a purified flavin-containing monooxygenase from pig and mouse liver [10, 19]. Our conclusions on the importance of flavin-containing monooxygenase for TMA metabolism by rat liver microsomes differ from conclusions obtained from studies on the cytochrome P450-dependent metabolism of TMA by yeast [20] and indicate that cytochrome P450-catalyzed TMA oxidation [15] does not apply to rat cytochrome P450 enzymes or, apparently, to pig and mouse microsomal enzymes. Although the metabolism of TMA by yeast microsomes was sensitive to inhibition by carbon monoxide, liver microsomes from untreated rats or from rats pretreated with pregnenolone 16α-carbonitrile were not inhibited by carbon monoxide (80% CO/20% O_2; Fig. 3). Since N-demethylation of tertiary amines is an unusual reaction for FMO, a possibility of another thermolabile enzyme catalyzing N-demethylation of TMA cannot be excluded entirely.

An interesting aspect of our studies was a 5-fold lower K_m for TMA metabolism may explain the more extensive metabolism of TMA to TMAO than to DMA *in vivo*. Our results also suggest the possibility that separate flavin/containing monooxygenases catalyze the N-oxygenation and demethylation of TMA. The possibility of separate flavin-containing monooxygenases that are under different regulatory control is supported by studies indicating that changing the atmosphere from air to 100% oxygen increased N-oxygenation activity but not TMA demethylation activity (Fig. 3) and that pretreatment of rats with pregnenolone 16α-carbonitrile increased TMA demethylation, but did not influence N-oxygenation of TMA (Fig. 2). It is noteworthy that the defect in TMA oxidation to TMAO in patients with fish-odor syndrome (trimethylaminuria) co-segregated with defective N-oxidation of nicotine, but not with the N-oxidation of verapamil or pinacidil [21, 22]. The lack of co-segregation could be due to different K_m values for the different substrates or separate enzyme systems responsible for N-oxidation of these drugs.

The small to moderate stimulatory effect of pregnenolone 16α-carbonitrile on the metabolism of TMA to DMA is of interest, although the overall metabolism of TMA was not increased significantly. The stimulatory effect of pregnenolone 16α-carbonitrile on TMA demethylation may result from a selective induction of one or more flavin

monooxygenases. It should be noted that steroid hormones have been reported to increase flavin-containing monooxygenase activity [23]: treatment of rats with prednisolone stimulates the N-oxidation of *N,N*-dimethylaniline in liver and lung, and treatment of mice with progesterone increases *N,N*-dimethylaniline N-oxidation in mouse liver.

Our observation of a 5-fold lower K_m value for TMA N-oxygenation than for N-demethylation and the lower rate of DMA than TMAO formation, even at near-maximum velocities, together with the observation that only about 39% of DMA administered to rats is excreted in urine within 24 hr [24] seems to agree well with the observation that humans given TMA excreted 95% of the dose as TMAO and less than 1% as DMA [2].

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