Endogenous formation of dimethylamine

Steven H. ZEISEL,*† Kerry-Ann DAcOSTA* and James G. FOX†

*Nutrition-Pathology Unit, Departments of Pathology and Pediatrics, Boston University School of Medicine, 85 East Newton Street (Room M1002), Boston, MA 02118, U.S.A., and †Department of Applied Biological Science, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, U.S.A.

An understanding of the biosynthesis and metabolism of dimethylamine (DMA) is important because it is a precursor of dimethylnitrosamine (nitroso-DMA). DMA is the major short-chain aliphatic amine in human and rat urine. DMA is formed from trimethylamine (TMA), which, in turn, is a breakdown product of dietary choline. Enzymes within gut bacteria catalyse both of these reactions; it is not known whether mammalian cells can form DMA. To determine the relative importance of dietary choline, bacteria and other mechanisms for the formation of DMA, we measured DMA excretion in the urine of rats fed on a diet devoid of choline, and in urine of rats with no bacterial colonization of the intestines. We also describe an improved gas-chromatographic method for the measurement of methylamines in biological fluids. In control rats there were significant amounts of DMA within several biological fluids [urine, 54.2 ± 3.0 $\mu\text{mol/kg}$ body wt. per 24 h (556.2 ± 37.5 nmol/ml); blood, 18.8 ± 1.9 nmol/ml; gastric juice, 33.5 ± 10.5 nmol/ml; means $\pm$ S.E.M.]. Animals eating a diet containing no choline excreted as much MMA and DMA as did choline-supplemented rats ($25\text{--}35$ $\mu\text{mol/kg}$ per 24 h), and they excreted slightly less TMA (2 versus 2.5 $\mu\text{mol/kg}$ per 24 h). Rats with no gut bacteria excreted the same amount of DMA in their urine as did the control animals ($45\text{--}55$ $\mu\text{mol/kg}$ per 24 h). They excreted much less MMA (16.3 ± 1.5 versus 40.3 ± 2.6 $\mu\text{mol/kg}$ per 24 h; mean $\pm$ S.E.M.; $P < 0.01$), TMA (0.7 ± 0.2 versus 2.5 ± 0.5 $\mu\text{mol/kg}$ per 24 h; mean $\pm$ S.E.M.; $P < 0.01$) and piperidine (2.0 ± 0.3 versus 6.3 ± 0.6 $\mu\text{mol/kg}$ per 24 h; mean $\pm$ S.E.M.; $P < 0.01$) in their urine. From our studies we conclude that DMA is present in significant amounts within gastric fluid, an environment that is ideal for nitrosamine formation (under acidic conditions, nitroso-DMA is chemically formed by the reaction of nitrite with DMA). Results also indicate that dietary choline was not the sole precursor for DMA formation and that gut bacteria are not essential for the formation of DMA. Hence in mammals there must be endogenous pathways that are capable of forming DMA; however, these endogenous mechanisms remain unidentified.

INTRODUCTION

An understanding of the biosynthesis and metabolism of DMA is important because it is a precursor of dimethylnitrosamine (nitroso-DMA). Nitrosamines can act as potent carcinogens in a wide variety of animal species, and there is no reason to assume that humans are resistant (Bartsch & Montesano, 1984). Although a causal relationship between nitrosamine exposure and cancer in humans has not been rigorously proved, epidemiological studies linking nitrosamines in tobacco products to oral cancer in humans strongly suggest such an association (Bartsch & Montesano, 1984).

DMA is the major short-chain aliphatic amine in human and rat urine (Asatoor & Simenhoff, 1965). Seafood contains significant quantities of methylamines because DMA and TMA are common degradation products of trimethylamine oxide, an end product of nitrogen metabolism in fish (Lundstrom & Racicot, 1983; Singer & Lijinsky, 1976). DMA can be formed from dietary choline, in a reaction catalysed by enzymes within gut bacteria (Zeisel *et al.*, 1983; Asatoor & Simenhoff, 1965). Rats that have been treated with neomycin to decrease bacterial colonization of gut continue to excrete some DMA (Asatoor & Simenhoff, 1965). Though this suggests that there may be endogenous mechanisms for its biosynthesis, remaining bacteria could still account for

DMA production. To determine the relative importance of dietary choline, bacteria and other mechanisms for formation of DMA, we measured DMA excretion in the urine of rats fed on a diet devoid of choline, and of rats with no bacterial colonization of the intestines. We also describe an improved method for the measurement of methylamines in biological fluids.

METHODS

Experimental protocols

In experiments to determine the rate of elimination of DMA into urine, four male Sprague-Dawley rats were given 330 μmol of DMA/kg body wt. by orogastric intubation. Animals were placed in individual metabolic cages, and urine was collected for two 24 h periods.

In the choline-deficiency study, rats were fed on the choline-supplemented (control) semi-synthetic diet for 1 week (Table 1). They then were randomly assigned to one of two groups; five rats were fed on the choline-deficient diet, and five rats were continued on the choline-supplemented diet for 4 more weeks. At the end of this period, all animals ate the choline-supplemented diet for 1 week. Animals were pair-fed, with calculations made on a daily basis. For one 24 h period of each study week, rats were placed in a metabolic cage and urine was collected.

Abbreviations used: MMA, monomethylamine; DMA, dimethylamine; TMA, trimethylamine.

† To whom correspondence and reprint requests should be addressed.

At the end of this 24 h period, the rats were anaesthetized with diethyl ether and blood was drawn by intracardiac puncture.

In the germ-free experiment, rats with no bacterial colonization of the bowel were used. They were fed on autoclaved rat chow, and urine was collected, into a sterilized collection vessel containing 6 M-HCl, for a single 24 h period. Control rats with normal bacterial flora were treated identically, but were not housed under aseptic conditions. At the end of the study a sterile swab was used to culture the colon contents. Animals were anaesthetized with CO₂ or light diethyl ether anesthesia and blood was drawn by intracardiac puncture. The animals were then exsanguinated. An abdominal incision rapidly exposed the stomach, and gastric juice was collected by direct aspiration.

Animals

In the choline-deficiency experiments we used young male Sprague-Dawley rats (125 g body wt.; Charles River Breeders, Wilmington, MA, U.S.A.). They were fed on a semi-synthetic diet (Table 1) and water *ad libitum*. They were housed in suspended stainless-steel wire cages, exposed to light during 08:00–18:00 h daily, in a climate-controlled room (24 °C).

In the germ-free experiments young male Fisher rats (100 g body wt.; Charles River Breeders) were used. These animals had been delivered by caesarean section under aseptic conditions, and subsequently were housed in germ-free facilities. They were shipped in specially filtered germ-free containers, and, on arrival, were housed in autoclaved metabolic cages in 'clean rooms' with reverse-flow laminar air exchange. Rats were exposed to light during 08:00–18:00 h daily, and the room was climate-controlled (24 °C). Autoclavable diet (Charles River rat/mouse/hamster formula) and water were autoclaved before use. No aerobic or anaerobic organisms were detected in rectal cultures taken from germ-free rats at Charles River Laboratories, or in cultures of germ-free-rat colon contents at our own diagnostic laboratory. Samples were cultured on tryptose/blood/agar with 5% (v/v) defibrinated sheep blood for 4 days at 37 °C and then for 3 days at 22 °C, on Shadler's agar with 5% (v/v) defibrinated sheep blood anaerobically at 35 °C, on nutrient/dextrose/agar for 7 days at 22 °C, on Klebsiella broth with 0.5% dextrose at 37 °C, on fluid thioglycollate broth for 3 days at 30 °C, and on fluid thioglycollate broth for 7 days at 55 °C. No bacteria were seen on microscopic examination of smears of intestinal samples stained with Crystal Violet. Animals used as controls were male Fisher rats (100 g body wt.; Charles River Breeders) delivered vaginally and housed in non-sterile facilities.

Diets

A semi-synthetic diet (Table 1) was employed in the choline-deficiency experiments; it contained soya-bean protein, which was low in methionine content. This minimized endogenous formation of choline by the methylation of phosphatidylethanolamine. Cystine supplement was added to meet the animal's sulphur amino acid requirement (Byington *et al.*, 1972). By our analysis the choline-deficient diet contained less than 5 µg of choline/g wet wt. and the choline-supplemented diet contained 706 µg of choline/wet wt.

The commercial rat chow (Charles River rat/mouse/

Table 1. Diets used in choline-deficiency experiment

The components listed below were mixed and added to a solution of boiled agar agar (Bacto Agar; Difco Laboratories, Detroit, MI, U.S.A.) (35 g/l of distilled water).

Ingredient	Content (% of dry wt.)
Soya-bean protein (grade II)*	6.0
Casein (vitamin-free)*	6.0
Dextrin (technical grade)*	48.5
Corn oil (Mazola)†	5.0
Hydrogenated fat (Crisco)‡	30.0
AIN mineral mixture no. 76*	3.5
AIN vitamin mixture no. 76 (choline-free)*	1.0
Cystine§	0.1
Choline chloride (control diet only)	0.2

* U.S. Biochemical Corp., Cleveland, OH, U.S.A.

† Best Products, Englewood Cliffs, NJ, U.S.A.

‡ Proctor and Gamble, Cincinnati, OH, U.S.A.

§ ICN Nutritional Biochemicals, Cleveland, OH, U.S.A.

|| Anachemia Chemicals, Montreal, Que., Canada.

hamster formula) comprised 23% protein, 4.5% fat and 72% carbohydrate, and by our analysis contained 1.83 mg of choline/g dry wt.

No methylamines were detected in an HClO₄ extract of 100 mg of either of the diets.

Analysis of methylamines

MMA, DMA, TMA and piperidine were measured by using a modification of the method of DiCorcia & Samperi (1974). A 2 ml sample of blood was immediately injected into a stoppered tube containing 2 ml of 10% (w/v) trichloroacetic acid. Tubes were centrifuged at 3000 g for 5 min at –4 °C, and the clear supernatant was aspirated and used for the methylamine assay. Samples of urine or gastric juice were collected into HClO₄ (so that final concentration was approx. 4% acid).

A portion of the acidified biological fluid (urine, gastric juice or blood extract) was placed into a sealed vial (Teflon-lined WISP septum; Waters Instruments, Milford, MA, U.S.A.). Propan-2-ol was added into each vial, along with an internal standard of isopropylamine. KOH (65%, w/v) was injected into the vial to bring the pH above 9. Vials were vigorously shaken, heated at 60 °C for 30 min, and then centrifuged at 1000 g for 5 min at –4 °C. A portion was drawn up into a syringe, followed by 1 µl of air and 1 µl of 30% (w/v) NH₃. This was injected into the glass-lined injection port (pretreated with KOH to inactivate any binding sites for methylamines) of the gas chromatograph (Sigma 300; Perkin-Elmer, Norwalk, CT, U.S.A.). We used a 2 m × 2 mm (internal diam.) glass chromatography column packed with 60/80 Carbowax B/0.8% KOH on 4% Carbowax 20 M (Supelco, Bellefonte, PA, U.S.A.), and eluted methylamines with He (25 ml/min) and a temperature gradient (75 °C for 2 min, then rising to 200 °C at 32 °C/min). Amines were detected with nitrogen/phosphorus detector (NPD; Perkin-Elmer), and peaks were integrated by using a computing integrator (LCI-100; Perkin-Elmer). During each sample run, we injected 20 µl

Fig. 1. Gas-chromatographic separation of methylamines

Methylamines were measured by using a gas-chromatography method. Samples of known standards or of urine were collected into HClO_4 (so that final concentration was approx. 4% acid). A portion of the acidified biological fluid (urine, gastric juice or blood extract) was placed into a sealed vial (Teflon-lined WISP septum). Propan-2-ol was added into each vial, along with an internal standard of isopropylamine. KOH (65%) was injected into the vial so as to bring the pH above 9. Vials were vigorously shaken, heated at 60 °C for 30 min and then centrifuged at 1000 *g* for 5 min at -4 °C. A portion was drawn up into a syringe, followed by 1 μl of air and 1 μl of 30% NH_3 . This was injected into the glass-lined injection port (pretreated with KOH to inactivate any binding sites for methylamines) of the gas chromatograph (Sigma 300). We used a 2 m $\times$ 2 mm (internal diam.) glass chromatography column packed with 60/80 Carbowax B/0.8% KOH on 4% Carbowax 20 M, and we eluted methylamines with He (25 ml/min) and a temperature gradient (75 °C for 2 min. then rising to 200 °C at 32 °C/min). Amines were detected with a nitrogen/phosphorus detector (NPD), and peaks were integrated by using a computing integrator (LCI-100). Time of peak elution (min) is indicated above each peak. Abbreviations: IPA, isopropylamine; Prop, propanol; Pyr, pyrrolidine; Pip, piperidine.

of 30% NH_3 on to the hot (150 °C) column as it was heated to 200 °C. This markedly decreased 'ghosting' of methylamines on subsequent runs. A typical chromatogram is presented in Fig. 1.

This method had a precision (100 $\times$ s.d./mean) for MMA of 1.6%, for DMA of 2.2%, for TMA of 2.0% and for piperidine of 3.2%. The assay was linear for amine contents of up to 1600 pmol, with a detection limit of less than 1 pmol. Recovery of methylamines added to urine samples was 83.3% for MMA, 90.8% for DMA, 98.7% for TMA and 104.6% for piperidine. Ammonia, ethylamine, diethylamine, propylamine, isopropylamine, benzylamine, creatinine, carnitine, betaine, choline, ethanolamine, pyrrolidine and phosphatidylcholine did not interfere with the assay when they were present at concentrations 10–100 times those of the amines of interest.

For selected samples, the identity of DMA was confirmed by using gas chromatography with mass-spectrometric detection. The acidified biological fluid was placed in the middle well of a three-well diffusion chamber (Conway & O'Malley, 1942), 0.05 M- H_2SO_4 was placed in the centre well, and 0.1 M-KOH saturated with K_2CO_3 was added to the outer and middle wells (the biological fluid and the base were mixed after the chamber was sealed). The sealed chamber was incubated at 50 °C for 4 h. During this time, methylamines were trapped in the acid-containing well (recovery of TMA was 97%, of DMA was 93% and of MMA was 70%). The acid was placed in a vial, adjusted to a volume of 200 μl with 0.1 M-HCl, and 10 mg of NaNO_2 was added. The sample was incubated at room temperature for 1 h, forming dimethylnitrosamine (nitroso-DMA). A 1 μl portion was injected on to a methylsilicone-bonded-phase fused silica capillary column (30 $\times$ 0.25 mm internal diam.; He carrier gas flow of 1.5 ml/min; Hewlett-Packard) in a Hewlett-Packard 5996 gas chromatograph-mass spectrometer. We used electron ionization and single-ion monitoring of $m/z = 74$, the molecular ion of nitroso-DMA. We analysed four urine specimens from germ-free rats and four urine specimens from choline-deficient rats (week 4), and identified a co-eluted peak with m/z 74 in quantities approximately equivalent to those predicted from the amounts of DMA determined by using the gas chromatograph-nitrogen/phosphorus detector assay. We observed only those ions known to be associated with authentic nitroso-DMA in the peaks eluted from the urine specimens (Fig. 2).

Choline assay

Choline was measured by using a radio-enzymic procedure (Goldberg & McCaman, 1973; McCaman & Stetzler, 1977). Food samples were extracted by homogenization in cold 15% (v/v) 1 M-formic acid in acetone. The homogenate was centrifuged at 2000 *g* for 10 min at 4 °C, and a portion of the supernatant was freeze-dried. This was resuspended in 10 mM-sodium phosphate buffer, pH 6.6. Plasma samples were diluted with 4 vol. of the phosphate buffer. Choline was isolated by liquid cation-exchange with tetraphenylboron in heptanone (5 mg/ml). It was then back-extracted into 0.4 M-HCl and freeze-dried. A buffer solution [0.1 M-glycylglycine/dithiothreitol (10 mg/ml)/1 M- MgCl_2 /0.1 M-ATP/water/choline kinase (0.5 unit/ml), 1:1:0.15:0.1:6:2, by vol.] was prepared and was preincubated at 30 °C for 15 min to remove any

Fig. 2. Confirmation of the identity of DMA by using gas chromatography-mass spectrometry

For a urine sample from a choline-deficient rat, the identity of DMA was confirmed by using gas chromatography with mass-spectrometric detection. The acidified biological fluid was placed in the middle well of a three-well diffusion chamber, 0.05 M-H₂SO₄ was placed in the centre well, and 0.1 M-KOH saturated with K₂CO₃ was added to the outer well and middle wells. The sealed chamber was incubated at 50 °C for 4 h. The acid from the centre well was placed in a vial, adjusted to a volume of 200 µl with 0.1 M-HCl, and 10 mg of NaNO₂ was added. The sample was incubated at room temperature for 1 h, forming dimethylnitrosamine (nitroso-DMA). A 1 µl portion was injected on to a methylsilicone-bonded-phase fused silica capillary column (30 m × 0.25 mm internal diam.; He carrier gas flow of 1.5 ml/min; Hewlett-Packard) in a Hewlett-Packard 5996 gas chromatograph-mass spectrometer. We used electron ionization and single-ion monitoring of $m/z = 74$, the molecular ion of nitroso-DMA. (a) Total-ion-current chromatograph of urine sample; (b) mass spectrum from known standard of nitroso-DMA; (c) mass spectrum from the single peak at 3.23 min of chromatograph of rat urine.

contaminating choline. [³²P]ATP (New England Nuclear; Boston, MA, U.S.A.) was added to the mixture. Samples were resuspended in this solution, and were incubated at 30 °C for 15 min. The resulting [³²P]phosphocholine that was formed was isolated by using an ion-exchange resin (Dowex AG-1 X8; Bio-Rad Laboratories, Richmond, CA, U.S.A.), and was eluted with 75 mM-ammonium acetate buffer, pH 10. Radioactivity was measured by using scintillation spectrophotometry (Rack Minibeta; LKB instruments, Gaithersburg, MD, U.S.A.). A [³H]choline internal standard was used to correct for sample recovery, which typically exceeded 70%.

Statistics

Data were analysed by one-way analysis of variance and Sheffe's test, and by paired *t* test.

RESULTS AND DISCUSSION

We have improved the existing methods for quantification of methylamines in biological samples. The extraction procedure that we describe removed many unwanted contaminants, and isolated the methylamines in a solvent that was eluted from the chromatographic column after the amines of interest. The use of a nitrogen/phosphorus detector, rather than a flame-ionization detector, increased the sensitivity and specificity of the assay. Our method for the measurement of amines in blood, urine and gastric juice provided rapid and reproducible results (Fig. 1). We were able to confirm the identity of DMA in selected samples by using mass spectrometry (Fig. 2) and h.p.l.c.

In control rats we found significant amounts of DMA within several biological fluids [urine, 54.2 ± 3.0 µmol/kg body wt. per 24 h (556.2 ± 37.5 nmol/ml); blood,

18.8 ± 1.9 nmol/ml; gastric juice, 33.5 ± 10.5 nmol/ml; means $\pm$ S.E.M.]. There was no difference in the DMA, MMA or TMA content of biological fluids between male Fisher and Sprague-Dawley rats. Rats given DMA (329 µmol/kg body wt.) excreted much of it in their urine ($39 \pm 6\%$ during the first 24 h after the dose, $6 \pm 0.3\%$ during the next 24 h; means $\pm$ S.E.M.). DMA was probably also excreted in the breath and faeces. For this reason, data on urinary excretion of DMA provide an indirect measure of rates of DMA synthesis. Rats eating chow always excreted more MMA and DMA in their urine than did rats eating the semi-synthetic diet (Table 2). Older rats excreted less MMA and DMA per kg body wt. than did lighter animals (Table 2). The amounts of DMA that we found in blood and urine are similar to those previously cited (Lowis *et al.*, 1983; Ishiwata *et al.*, 1984). To our knowledge, this is the first report demonstrating significant amounts of DMA in gastric fluid. Nitrite found in gastric fluid is formed from dietary nitrate in the oral cavity by reactions mediated by bacterial enzymes (White, 1975; Tannenbaum *et al.*, 1976). Nitroso-DMA is rapidly formed when DMA and nitrite are mixed in a test tube containing gastric juice (Lane & Bailey, 1973), and *in vivo* in the dog stomach (Lintas *et al.*, 1982). Thus the demonstration of DMA in gastric fluid is of special interest, as it completes the conditions necessary for the formation of nitrosamines.

Choline, a quaternary amine, is a component of the normal rat and human diet (Zeisel, 1981). DMA can be formed from choline, via the intermediate TMA, in a reaction mediated by gut bacteria (Asatoor & Simenhoff, 1965; Zeisel *et al.*, 1983). We deprived rats of choline for more than 4 weeks, and found that they continued to excrete normal amounts of DMA and MMA (Fig. 3). We observed that TMA excretion in the choline-deficient rats

Fig. 3. Methylamine excretion by choline-deficient rats

Rats were fed on the choline-supplemented semi-synthetic diet for 1 week. They then were randomly assigned to one of two groups: five rats were fed on the choline-deficient diet and five rats were continued on the choline-supplemented diet for 4 more weeks. At the end of this period, all animals ate the choline-supplemented diet for 1 week. Animals were pair-fed, with calculations made on a daily basis. For one 24 h period of each study week, rats were placed in a metabolic cage and urine was collected. Methylamines in urine were determined by using a gas-chromatography method described in the text. Data are expressed as means $\pm$ S.E.M. ●, Control group; ○, choline-deficient group. ** $P < 0.01$; * $P < 0.05$.

Table 2. Effects of diet, strain and body weight on urinary excretion of DMA in rats

Rats were placed in a metabolic cage and urine was collected for a 24 h period. Methylamines in urine were measured by using a gas-chromatography method described in the text. Data are expressed as means $\pm$ S.E.M. Abbreviation: SSD, choline-supplemented semi-synthetic diet.

Strain (n)	Body wt. (g)	Diet	Urine amine ($\mu\text{mol/kg per 24 h}$)		
			MMA	DMA	TMA
Sprague-Dawley (8)	100	Chow	36.4 ± 1.8	61.6 ± 2.4	1.5 ± 0.2
Sprague-Dawley (5)	300	Chow	$27.7 \pm 2.5^{**}$	$44.2 \pm 2.5^{**}$	1.6 ± 0.3
Fisher (12)	100	Chow	38.3 ± 2.3	53.7 ± 2.6	2.2 ± 0.4
Sprague-Dawley (10)	100	SSD	$8.2 \pm 1.0^{**}$	$31.0 \pm 2.8^{**}$	2.0 ± 0.4

** $P < 0.01$, different from Sprague-Dawley rats (100 g) eating chow.

was significantly lower than in the control animals (Fig. 3). This would be expected if choline were a significant source of TMA. The choline-deficient and choline-supplemented rats gained equivalent amounts of weight during the 6 weeks of the study (184 ± 8 g; mean $\pm$ S.E.M.). The experimental animals were choline-depleted, as plasma choline concentrations in the choline-deficient rats fell sharply during the first day on the diet (from 16.2 ± 1.0 to 9.9 ± 1.1 nmol/ml; means $\pm$ S.E.M.), and reached their lowest values within 4 days on the diet (6.0 ± 0.5 nmol/ml; mean $\pm$ S.E.M.). Plasma choline concentrations of the choline-deficient rats and of the control rats were the same when all animals were fed with the choline-supplemented diet during weeks 5–6 of the study (12.5 ± 1.6 nmol/ml; mean $\pm$ S.E.M.). These data show that dietary choline is not essential for the formation of dimethylamine. It is possible that some choline might still have been delivered to gut bacteria, e.g. choline derived from digestion of sloughed intestinal cells, from bile and

from synthesis *de novo* by gut bacteria. It is unlikely that such sources could have replaced the large amounts of choline normally present in the diet, and therefore these sources could not have been responsible for the almost-normal amount of DMA excreted by choline-deficient rats.

There was a gradual decline in the amount of DMA/kg per 24 h excreted by both choline-supplemented and choline-deficient rats during the 6 weeks of the study (Fig. 3). In other experiments we observed that 300 g rats excreted less MMA and DMA per kg body wt. than did 100 g rats (Table 2). However, we found that excretion/body weight was far more uniform than was excretion/urinary creatinine or excretion/time. Perhaps excretion/surface area would have been more appropriate, but, as we always compared matched groups of similar body weight, the method for data normalization did not influence the interpretation of the data. Animals eating rat chow always excreted more MMA and DMA than did

Fig. 4. Methylamine excretion by germ-free rats

Rats with no bacterial colonization of the bowel, and control rats with normal bacterial flora, were housed in autoclaved metabolic cages, and urine was collected for a single 24 h period. Animals ingested rat chow that was methylamine-free. Methylamines in urine were determined by using a gas-chromatography method described in the text. ■, Controls ($n = 8$); ▨, germ-free ($n = 7$). Data are expressed as means $\pm$ S.E.M. ** $P < 0.01$, different from control.

animals eating the semi-synthetic diet. We determined that the chow contained no MMA, DMA or TMA, and that it contained approximately the same amount of choline as the semi-synthetic diet. It is possible that the chow contained other dietary precursors for formation of methylamines (possibly carnitine, sarcosine or creatinine). If this is the case, our data suggest that these components of the diet might contribute 10 $\mu\text{mol/kg}$ per 24 h to urinary excretion of DMA in the rat (Fig. 3 and Table 2).

It is impossible to prove the absolute absence of gut flora. However, since we were not able to culture organisms, it is safe to assume that the germ-free rats were no more than minimally colonized with gut bacteria. We found that the presence of gut bacteria was not essential for the formation and excretion of DMA in rats; DMA excretion was the same in germ-free and control animals (Fig. 4). The source of this DMA in germ-free rats is unknown, as it had been assumed that the enzyme activities needed to produce DMA are only present in bacteria (Zeisel *et al.*, 1983). It is possible that DMA was derived from breakdown of endogenous choline or choline-containing phospholipids, or that this DMA was formed *de novo* from simple precursors. The excretion of TMA and piperidine was markedly decreased in the

germ-free animals. Gut bacteria form TMA from dietary choline, and form piperidine exclusively from dietary lysine (Blau, 1961; Asatoor & Simenhoff, 1965). The decreases in TMA and piperidine excretion support our assumption that the rats had minimal colonization with bacteria. MMA excretion also was much diminished in germ-free animals, suggesting that much methylamine is formed via the action of gut bacteria.

In summary, we have shown that DMA is present in significant amounts within gastric fluid, an environment that is ideal for nitrosamine formation. We have demonstrated that dietary choline is not the sole precursor for DMA, and that gut bacteria are not essential for DMA formation. We conclude that in mammals there must be endogenous pathways that are capable of forming DMA; however, these endogenous mechanisms remain unidentified.

This work was supported by Grant CA-26731 from the National Institutes of Health. We thank Dr. J. Wishnok for his assistance with the mass-spectrometric analyses.

REFERENCES

- Asatoor, A. M. & Simenhoff, M. L. (1965) *Biochim. Biophys. Acta* **111**, 384–392
- Bartsch, H. & Montesano, R. (1984) *Carcinogenesis* **5**, 1381–1398
- Blau, K. (1961) *Biochem. J.* **80**, 193–200
- Byington, M. H., Howe, J. M. & Clark, H. E. (1972) *J. Nutr.* **102**, 219–228
- Conway, E. J. & O'Malley, E. O. (1942) *Biochem. J.* **36**, 655–661
- DiCorcia, A. & Samperi, R. (1974) *Anal. Biochem.* **46**, 927–981
- Goldberg, A. M. & McCaman, R. (1973) *J. Neurochem.* **20**, 1–8
- Ishiwata, H., Iwata, R. & Tanimura, A. (1984) *Food Chem. Toxicol.* **22**, 649–653
- Lane, R. P. & Bailey, M. E. (1973) *Food Cosmet. Toxicol.* **11**, 851–854
- Lintas, C., Clark, A., Fox, J., Tannenbaum, S. R. & Newberne, P. M. (1982) *Carcinogenesis* **3**, 161–165
- Lewis, S., Eastwood, M. A. & Brydon, W. G. (1983) *J. Chromatogr.* **278**, 139–143
- Lundstrom, R. C. & Racicot, L. D. (1983) *J. Assoc. Off. Anal. Chem.* **66**, 1158–1163
- McCaman, R. & Stetzler, J. (1977) *J. Neurochem.* **28**, 669–671
- Singer, G. M. & Lijinsky, W. (1976) *J. Agric. Food Chem.* **24**, 550–553
- Tannenbaum, S. R., Weisman, M. & Fett, D. (1976) *Food Cosmet. Toxicol.* **14**, 549–552
- White, J. W. (1975) *J. Agric. Food Chem.* **23**, 886
- Zeisel, S. H. (1981) *Annu. Rev. Nutr.* **1**, 95–121
- Zeisel, S. H., Wishnok, J. S., Blusztajn, J. K. (1983) *J. Pharmacol. Exp. Ther.* **225**, 320–324

Received 25 April 1985/1 July 1985; accepted 24 July 1985