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N^G,N^G-Dimethyl-L-arginine, a dominant precursor of endogenous dimethylamine in rats

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Summary. The metabolic significance of N^G,N^G-dimethyl-L-arginine (DMA) as a precursor of endogenous dimethylamine (DMN) in rats was examined in connection with the wide distribution and active operation of dimethylargininase (EC3.5.3.18) in rat tissues (Kimoto et al., 1993). When [methyl-^{14}C]DMA was administered intraperitoneally to rats, the radioactive DMN was detected in various tissues as a major radioactive metabolite one hour after injection, and about 65% of the radioactivity administered was recovered in the first 12-h urine as DMN. In the case of the [^{14}C]DMN-injected rats, almost all the radioactivity was excreted in the 12-h urine as DMN, except for a negligible amount of radioactivity found in urea. The time-dependent decrease in the specific radioactivity of DMA and DMN in urine showed that dimethylargininase was significantly involved in the *in vivo* formation of DMN by the hydrolytic cleavage of DMA released from methylated proteins and that DMA is a dominant precursor of endogenous DMN in rats.

Keywords: Amino acids – Methylated arginines – N^G,N^G-Dimethyl-L-arginine – Dimethylargininase – Dimethylamine – *N*-Nitrosodimethylamine

Introduction

Guanidino-*N*-methylated arginines, such as N^G,N^G-dimethyl-L-arginine (DMA), N^G,N'^G-dimethyl-L-arginine (DM'A) and N^G-monomethyl-L-arginine (MMA), are widely distributed in proteins methylated post-translationally (Paik and Kim, 1975) and are continously released in body fluids during *in vivo* breakdown of the proteins (Kakimoto and Akazawa, 1970). We previously demonstrated that the metabolic pathways of methylated arginines are actively working in rats (Ogawa et al., 1987a). Subsequently, we have found a unique enzyme, dimethylargininase (EC3.5.3.18) (Ogawa et al., 1987b; Ogawa et al.,

1989), which hydrolyzes DMA and MMA to form L-citrulline and dimethylamine (DMN), and L-citrulline and monomethylamine (MMN), respectively, and that the enzyme occurs widely and operates actively in rat tissues (Kimoto et al., 1993).

These findings led us to assume that endogenous DMN may be formed mainly from DMA. DMN is a major aliphatic amine found in human and rat urine (Asatoor and Simenhoff, 1965). It has been considered that endogenous DMN can be formed from dietary choline by gut bacteria (Asatoor and Simenhoff, 1965; Zeisel et al., 1983). Some researchers, however, pointed out that dietary choline was not a sole precursor for DMN and that gut bacteria were not essential for the formation of DMN (Asatoor and Simenhoff, 1965; Zeisel et al., 1985). Zeisel et al., (1985) suggested that there might be another dominant pathway involved in the formation of endogenous DMN, but this idea remains unproved. It is very important to elucidate the mechanism of the *in vivo* formation of DMN because this amine is known as a precursor of *N*-nitrosodimethylamine, a potent carcinogen (Magee and Barnes, 1956; Haas et al., 1973). In the present study, we aimed to elucidate whether the hydrolytic cleavage of DMA by dimethylargininase is a dominant pathway for the formation of endogenous DMN in rats using [methyl-^{14}C]DMA and [^{14}C]DMN, respectively, and also discussed as to the possibility of the *in vivo* nitrosation of DMN.

Materials and methods

Materials

[^{14}C]Dimethylamine hydrochloride (58 Ci/mol) was purchased from Amersham International plc (Buckinghumshire, England). The other chemicals and reagents were analitical grade.

Preparation of radioactive DMA

[^{14}C]Dimethylamine hydrochloride (250 μCi) was diluted with unlabeled dimethylamine hydrochloride (20 mmol) and placed in a sealed tube containing aqueous solution of urea (1 mmol). The mixture was heated at 140°C for 1 h in an oil bath. The reaction product was purified using a column of Dowex 50 W × 4 (H^+ form) and crystallized in hot ethanol. Recrystallized [methyl-^{14}C]dimethylurea gave m.p. 179–181°C. Analytical data calc. for $C_3H_8N_2O$: C, 40.90; H, 9.15; N, 31.79%. Found: C, 40.75; H, 9.60; N, 32.33%.

[methyl-^{14}C]DMA was synthesized by coupling the copper complex of L-ornithine with [methyl-^{14}C]dimethylurea according to the method of Kakimoto and Akazawa (1970). The radioactive compound (specific activity; 12.5 mCi/mol) was chromatographically pure and was used for the metabolic experiments described below.

Animals and metabolic experiments

Male rats of Wistar strain (average body weight, 170 g) were transferred individually into a glass metabolic cage and fed the normal diet (Sasaoka et al., 1976) and water ad libitum. They were divided into three groups. Two groups (four rats per group) were injected intraperitoneally with 5 μmol of [methyl-^{14}C]DMA (12.5 mCi/mol) or 0.17 μmol of [^{14}C]DMN (58 mCi/mol). Urine was collected in a glass flask containing 5 ml of 0.1 M HCl at 12-h intervals and expired CO_2 was continously absorbed into a mixture of monoethanolamine/methylcellosolve (1:2, v/v) (Jeffay and Alvarez, 1961). The other group

injected with [methyl-^{14}C]DMA in the same manner as described above was used for the preparation of the tissue samples and plasma. The rats were etherized at 1 h after the injection of the radioactive compound and exsanguinated by heart puncture with heparinized syringes. Tissues were removed immediately and homogenized with equal volume of ice cold water and subsequently with two volumes of 10% trichloroacetic acid (TCA) using a Potter-Elvehjem glass homogenizer. The homogenates were centrifuged at 10,000 × *g* for 20 min and the supernatants were used as TCA-extract. Blood was centrifuged at 1,500 × *g* for 10 min and the plasma was mixed with equal volume of 10% TCA and centrifuged in the same manner as described above. The supernatant obtained was used as TCA-extract.

Fractionation of radioactive metabolites

Urine samples (approximately pH 2.0) were applied to a column (1.5 × 40 cm) of Dowex 50 W × 4 (H^+ form). Elution was subsequently performed with water (400 ml), 0.5 M HCl (500 ml), and 2 M NH_4OH (250 ml). The tissue samples were chromatographed in the same manner as in the case of urine samples except for the procedure was scaled down.

Isolation and determination of DMN

According to the above mentioned procedure, authentic DMN was eluted in the 0.5 M HCl fraction. As shown in Figs. 1 and 2, peaks A and A′ appeared in the 0.5 M HCl fraction. These fractions were concentrated in vacuo and subjected to the following determination of DMN. DMN was spectrophotometrically determined according to the method of Beal and Bryan (1978) as follows. The cupric sulfate-ammonium solution (0.5 ml) and 1.5 ml of the carbon disulfide-benzene solution were added to 1 ml of the concentrated sample. The resultant nonmiscible phases were mixed on a Vortex mixer for 1 min, after which 0.25 ml of 30% acetic acid was added to the mixture and the phases were mixed again for 30 sec. The benzene layer, which contains copper dimethyldithiocarbamate (CDDC) derivative formed from DMN, was transferred to a cuvette and the absorbancy at 434 nm was measured with a Hitachi Model 200-20 spectrophotometer.

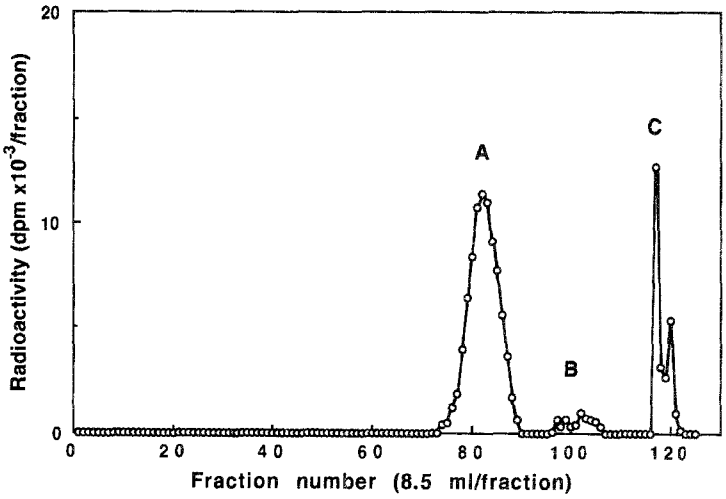

Fig. 1. Chromatographic separation of the radioactive metabolites in the urine sample of the [methyl-^{14}C]DMA-injected rat. A urine sample corresponding to half of the first 12-h urine was applied on the column. Peaks A-C correspond to unknown metabolite (compound A), N^{α}-acetyl-DMA, and a mixture of γ-(*N*,*N*-dimethylguanidino)butyric acid and unchanged DMA, respectively

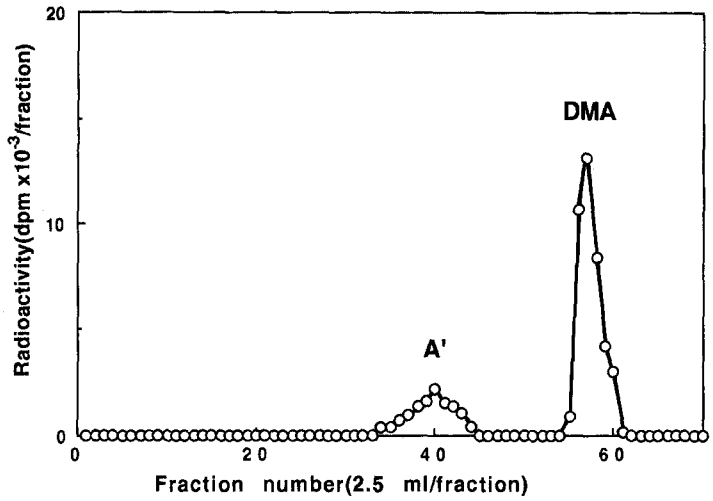

Fig. 2. Chromatographic separation of the radioactive metabolites in the kidney sample of the [methyl-^{14}C]DMA-injected rat. A tissue sample corresponding to 2.04 g of the tissue was applied on the column. Peak A′ contains DMN and unknown metabolites

Results

Behavior of [methyl-^{14}C]DMA in rats

The percentages of the radioactivity in the urine and expired CO_2 to that injected are shown in Table 1. Most of the radioactivity injected into rats were excreted in the urine during the first 12 h after injection and thereafter the excretion was quickly reduced. After 48 h, about 91% of the radioactivity of [methyl-^{14}C] DMA injected was excreted in the urine. These results indicate that only small portion of the radioactivity of [methyl-^{14}C]DMA injected was retained in the rat body. On the other hand, any radioactivity was not found in expired CO_2. These findings show that the metabolic fate of the radioactive carbon moiety of [methyl-^{14}C]DMA in rats is simpler than those of [1,2,3,4,5-^{14}C]DMA (Ogawa et al., 1987a).

Table 1. Recovery of the radioactivity in urine and expired CO_2

Time (h)[a]	Urine[b] (% of the radioactivity injected)	CO_2
0–12	89.9 ± 7.0	n.d.
12–24	1.0 ± 0.9	n.d.
24–36	0.11 ± 0.03	n.d.
36–48	0.05 ± 0.08	n.d.

n.d. not detected.
[a] Time after administration of [methyl-^{14}C]DMA.
[b] Mean ± SD (n = 4).

Identification of radioactive metabolites in urine

Figure 1 shows a chromatogram of the radioactive metabolites excreted in the urine after the intraperitoneal injection of [methyl-^{14}C]DMA. Three radioactive peaks (A, B, and C) were observed on the chromatogram, and 70% of the total radioactivity in the urine existed in peak A. Authentic DMN was eluted at position corresponding to peak A, suggesting that the radioactive compound in peak A is DMN. In order to confirm the identity, the copper dimethyldithiocarbamate (CDDC) salt of this radioactive compound in peak A (compound A) was prepared as described under *Materials and methods*. The radioactivity in peak A was quantitatively recovered in benzene layer as CDDC salt (99.2 ± 6.4%; Mean ± SD, n = 4). This result indicates that all the radioactivity in peak A existed as DMN. The behavior of CDDC derivative of compound A on thin-layer chromatography with various solvent systems were identical with those of the authentic derivative (Table 2), demonstrating that compound A is DMN. The minor peaks B and C are shown to contain N^{α}-acetyl-DMA and a mixture of γ-(*N*,*N*-dimethylguanidino)butyric acid and unchanged DMA, respectively, as described in the previous paper (Ogawa et al., 1987a).

Table 2. Thin-layer chromatographic behavior of the copper dithiocarbamate derivatives of compound A and authentic DMN

Solvent system[a]	R_f value[b]	
	Compound A	Authentic DMN
I	0.72	0.72
II	0.42	0.43
III	0.89	0.89

[a] Solvent system used: system I, chloroform/methanol (99:1, v/v); system II, carbon tetrachloride/acetonitrile (96:4, v/v); system III, methanol/dioxane (1:1, v/v).
[b] The values are expressed as the means obtained from 3 separate experiments.

Metabolites in tissues and plasma

The distribution of the radioactivity in the tissues and plasma was examined at 1 h after the injection of [methyl-^{14}C]DMA (Table 3). The radioactivity accumulated in various tissues, particularly in kidney and pancreas at high concentrations. The TCA-extracts of the tissue and plasma samples were fractionated in the same manner as described in the case of urine sample to characterize the radioactive metabolites. A typical chromatogram obtained with the kidney sample is shown in Fig. 2. In addition to the peak of unchanged DMA, peak A′ was observed on the chromatogram. The profiles of the radioactive metabolites in all the tissue samples except for the plasma sample were closely similar to that obtained with the kidney sample. No peak A′ was found only on the chromatogram obtained with the plasma sample. The main radioactive metabolite in peak

Table 3. Distribution of the radioactivity in the tissues and plasma of the [methyl-^{14}C]-DMA-injected rats

Tissue	Radioactivity[a] (dpm × 10^{-3}/g wet weight)
Kidney	8.62 ± 0.39
Liver	1.25 ± 0.18
Intestine	0.98 ± 0.09
Pancreas	4.44 ± 0.36
Stomach	1.11 ± 0.14
Heart	0.77 ± 0.03
Lung	1.00 ± 0.07
Spleen	1.34 ± 0.19
Plasma	1.60 ± 0.15[b]

[a] The radioactivity in the table expresses that in the TCA-extracts of the tissues and plasma. Results are presented by the mean ± SD (n = 3).
[b] dpm × 10^{-3}/ml of plasma.

Table 4. The radioactivity in DMN in the tissues and urine

Tissue[a]	DMN (dpm × 10^{-3}/g wet weight)	Specific radioactivity (dpm × 10^{-4}/μmol)
Kidney	2.22 ± 0.35[b]	1.6 ± 0.11[b]
Liver	0.29 ± 0.05[b]	1.5 ± 0.13[b]
Intestine	0.23[c]	1.4[c]
Stomach	0.33[c]	1.5[c]
Urine[d]	96.2 ± 12.0[b]	1.4 ± 0.10[b]

[a] DMN in other tissues such as pancreas, heart, lung, spleen could not be estimated because of the limited amounts of the tissue preparations. No radioactive DMN was found in the plasma sample.
[b] Mean ± SD (n = 3).
[c] The pooled sample of 3 rats was used.
[d] The urine collected for the first 12 h after the administration of [methyl-^{14}C]DMA.

A′ was identified as DMN and the radioactivity in DMN in the tissue and plasma samples were measured as its CDDC derivatives in peak A′ in the same manner as the urine sample. The distribution of radioactive DMN in rat tissues are summarized in Table 4. DMN accumulated in kidney at high concentration and also was detected in liver, stomach, and intestine at low concentrations. The specific radioactivities of DMN obtained from the tissue and urine samples were shown to be almost the same value and 60% in magnitude of that of [methyl-^{14}C]DMA injected in rats.

Fate of DMN

Furthermore, [^{14}C]DMN was intraperitoneally injected to investigate its fate in rat and the radioactive metabolites in the urine sample were characterized in

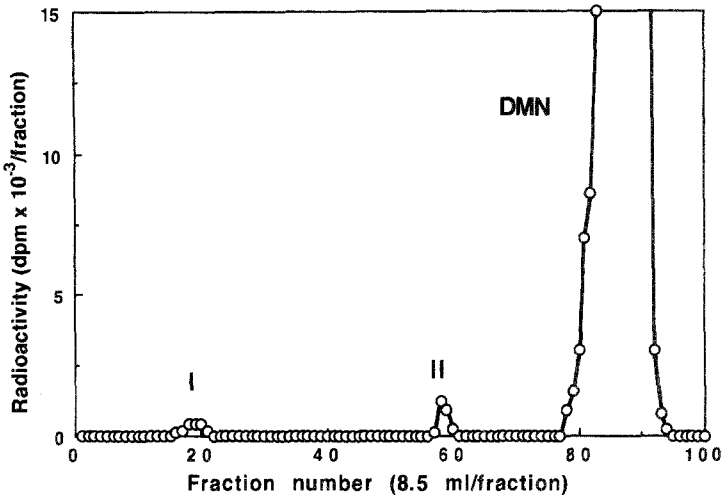

Fig. 3. Chromatographic separation of the radioactive metabolites in the urine sample of the [^{14}C]DMN-injected rat. A urine sample corresponding to one-tenth of the first 12-h urine was applied on the column

the same manner as those in the urine sample from the rats injected with [methyl-^{14}C]DMA. A large portion of the radioactivity was excreted in the first 12-h urine and only 0.5% of the radioactivity was detected in the CO_2 expired for the first 12 h. As shown in Fig. 3, most of the urinary radioactivity was retained in unchanged DMN. In addition, minor peaks I and II were observed on the chromatogram. The radioactive compound in peak I was suggested to be urea by comparison of the retention time on the chromatogram with authentic urea. The identity of the compund was confirmed by cochromatography followed by autoradiography (data not shown). However, the radioactive compound in peak II is so small that its identity remains unsolved. This result suggests that DMN is rapidly excreted without further appreciable metabolic changes.

Discussion

Since the methylated amino acids including N^{τ}-methyl-L-histidine have been assumed to be readily excreted in urine without further degradations in animals, the study for the biological roles of the methylated arginine residues in proteins and those in free form is little advanced . Recently, free DMA and MMA have been shown to act as competitive inhibitors of L-arginine: nitric oxide (NO) generating pathway (Hibbs et al., 1987a; Hibbs et al., 1987b; Sakuma et al., 1988). Furthermore, it has been shown that dimethylargininase newly identified in rat kidney can metabolize specifically DMA and MMA (Ogawa et al., 1989). As described above, [methyl-^{14}C]DMA administered into the rats released radioactive DMN which was excreted in the urine without undergoing any catabolic process (Figs. 1 and 3). The specific radioactivities of DMN (ca. 1.5×10^4 dpm/μmol) and DMA excreted in the 12-h urine were about 60% in magnitude of that of the injected [methyl-^{14}C]DMA (2.7×10^4 dpm/μmol) (Table 4). On the other hand, the radioactive DMN excreted in the 1-h urine exhibited 2.1 $\times$

10^4 dpm/μmol, about 80% of the specific radioactivity of injected [methyl-^{14}C] DMA. Thus, the time-dependent decrease in the specific radioactivity of DMN suggests that [methyl-^{14}C]DMA administered may be largely diluted with endogenous DMA continuously liberated from methylated proteins. In preliminary experiment, we investigated the DMA concentrations in various rat tissues. The amounts of free DMA in kidney, brain, and liver were 35, 3.25, and 1.5 nmol/g wet weight, respectively. In addition, the amounts of DMA residue in methylated proteins in liver, kidney, pancreas, and brain were 0.59, 0.29, 0.83, and 0.59 μmol/g protein. The above evidence indicates that DMA injected can be diluted with endogenous DMA. These findings strongly supported the idea that DMA may be a major source for endogenous DMN.

Previously, we suggested that DMA and MMA serve as endogenous inhibitors of the NO formation and that dimethylargininase may play a role in the regulation of the concentration of methylated arginines and the NO biosynthesis (Kimoto et al., 1993). This idea has been supported by other research groups (Hecker et al., 1990; Vallance et al., 1992). Thus, dimethylargininase is likely to operate positively for rising the concentrations of both DMN and NO in mammalian cells.

L-Arginine, the primary nitrogen source for NO synthesized by many cell types (Palmer et al., 1988; Bredt and Snyder, 1990; Yui et al., 1991a; Yui et al., 1991b), has been also shown to be a nitrogen source for nitrate synthesized in mammals (Leaf et al., 1989; Leaf et al., 1990). In some cases, immunostimulation by *Escherichia coli* lipopolysaccharide increases the biosynthesis of nitrate and nitrite (Leaf et al., 1989; Wagner et al., 1983; Iyengar et al., 1987). Miwa et al. (1987) have shown that activated macrophages can nitrosate secondary amines *in vitro*. These reports suggest that mammalian cells, especially immunostimulated cells may be capable of forming nitrosoamine even under the physiological conditions in the presence of nitric oxide. *N*-Nitrosodimethylamine, the nitrosation product of dimethylamine, is known as a potent carcinogen (Magee and Barnes, 1956; Haas et al., 1973) and is suggested to be formed in the field of NO generation (Hecht, et al., 1989). Therefore, we also examined whether DMN derived from DMA is metabolized to *N*-nitrosodimethylamine in rats. However, the formation of *N*-nitrosodimethylamine from DMN in normal rats could not be demonstrated (Fig. 3). This observation suggests that nitrosation of DMN is negligible at least in normal rats.

The present study provides useful knowledge in understanding the metabolism of dimethylamino moiety of DMA and an additional insight into the possible formation of nitrosodimethylamine in the immunologically activated rats.

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