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Metabolism of N^G-Monomethyl-L-arginine Formation of N-Monomethylurea in rat

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N^G-Monomethyl-L-arginine의 대사 : 흰쥐에서 N-monomethylurea의 생성

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국문초록

^{14}C로 표지된 N^G-mono[^{14}C-methyl]-L-arginine을 흰쥐에 경구투여하여 7일동안에 66.3%의 방사능이 회수되었다. 회수된 총방사능의 86.2%는 처음 24시간내에 배설되었으며, 그 분포는 산성물질, 염기성물질, 중성물질 및 회수된 N^G-monomethyl-L-arginine에서 각각 33.3%, 40.2%, 12.5% 및 0.3%이었다. 중성물질 방사능의 약 50%는 N-monomethylurea에 해당하며 이는 투여한 전체방사능의 6%이었다.

ABSTRACT

After oral administration of ^{14}C-labelled N^G-mono[^{14}C-methyl]-L-arginine into rats, 66.3% of the administered radioactivity has been recovered in the urine, and 86.2% of the total of the recovered radioactivity is recovered in the first 24-hr urine. Distributions of the radioactivity of the acidic, basic, and neutral portions and unmetabolized N^G-monomethyl-L-arginine are 33.3%, 40.2%, 12.5%, and 0.3%, respectively. The radioactivity corresponding N-monomethylurea is about 50% of the neutral portion and 6% of the administered radioactivity.

INTRODUCTION

Tumors have been produced by simultaneous feeding of mice and rats with sodium nitrite and N-monmethylurea[1], which results the formation of 1-N-methyl-1-N-nitrosourea[2]. N^G-monomethyl-L-arginine was formed by protein

methylase I (S-adenosyl-L-methionine: protein (arginine) N-methyltransferase, E.C.2.1.1.23) with S-adenosyl-L-methionine as methyl group donor[3,4].

After injection of N^G-monomethyl-L-arginine into rabbits, 0.14% of the injected amino acid was recovered and ornithine was found in 24 hr urine[5]. This pattern suggested that N^G-monmethyl-L-arginine was most likely hydrolyzed by an arginase-type enzyme to be formed ornithine and N-monomethylurea.

Formation of N-monomethylurea from N^G-monomethyl-L-arginine with tissue homogenates was found by colorimetric assay method using diacetyl monooxime as coloring reagent[6]. But the very similar absorption maximum of N-monomethylurea to those of the endogeneous contaminations (urea, alloxan, biuret, citrulline……) showed[7] that the colorimetric assay method was not suitable for conformation of the N-monomethylurea from the contaminations.

In the present paper, author presents evidence of the formation of the N-monomethylurea by thin-layer chromatography, cation-exchange resin column liquid chromatography, and scintillation spectrometry after oral administration of ^{14}C-labelled N^G-mono[^{14}C-methyl]-L-arginine into rats.

MATERIALS AND METHODS

Materials

Dowex 50(H^+), Dowex 1(Cl), p-dimethylaminobenzaldehde, flavianic acid, urea, and methyl iodide were purchased from Sigma Chem. Co., St. Louis, MO, and all other chemicals were from various commercial sources and were of the highest grade available. Mono[^{14}C] methylamine HCl (specific activity, 46.0 mCi/mmol) was purchased from New England Nuclear, Boston, MA. Sprauge-Dawley rats were fed *ad libitum* on rat chow, and all were housed at 20-25°C. N^G-Mono[^{14}C-methyl]-L-arginine was synthesized by coupling the copper complex of ornithine and ^{14}C-labelled N, S-dimethylthiopseudouronium iodide which was derived from N-mone[^{14}C-methyl]amine, CS_2, ammonia, and ethyl chloroformate[8]. The reaction product was purified by ion-exchange resin column chromatography and crystallization its flavianate from water. An aqueous solution of the free amino acid was obtained by mixing the flavianate and strong anion-exchange resin (OH^-) in water at room temperature. Purity was identified by thin-layer chromatography and thin-layer electrophoresis[9].

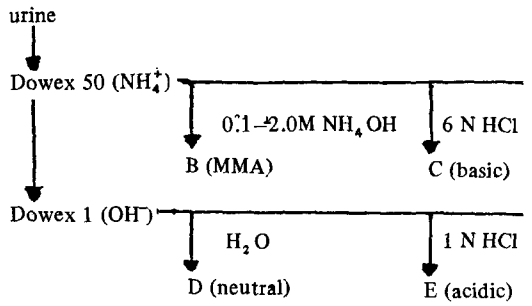
Methods

Preparation of urine samples

Sprague-Dawley rats weighing 200-250 g of male were used. An aqueous solution of N^G-mono[^{14}C-methyl]-L-arginine (9×10^5 dpm/0.5 ml) was orally administered into rats. The animal was maintained in metabolism cage on standard pellets and water *ad libitum* during the study, and 24-hr urine samples were collected in a 50 ml cylinder which was cooled in an ice-water bath for 7 days after administration. Aliquots of 0.5 ml of each urine sample were counted for radioactivity in 10 ml of scintillant at 4°C (Table 1).

Ion-exchange resin column chromatography of radioactive urine

The first 24-hr urine sample was centrifuged at 5,000 g for 30 min at 4°C. Aliquot (2.2×10^5



Scheme I. Separation of metabolites

dpm) of the supernatant and 1 ml of 25 mM monomethyl-L-arginine were applied to a column (1.37×6.8 cm) of Dowex 50 (×8; NH_4^+; 200-400 mesh) and eluted with 100 ml of water (eluent A). The column was then eluted with a gradient formed with 0.1-2.0 M NH_4OH (100 ml each) at a flow rate of 2 ml/min with 4 ml each fraction. Aliquots of every other fraction were spotted on filter paper, dried, and visualized with ninhydrin solution (0.2% ethanol solution) for detection of N^G-monomethyl-L-arginine. The fraction of the N^G-monomethyl-L-arginine were pooled, dried *in vacuo*, redissolved in 1 ml of water, and then counted for N^G-monomethyl-L-arginine (MMA) (B). The column was then washed with 100 ml of water, and eluted with 100 ml of 6 NHCl at flow rate of 1 ml/min. The eluents were pooled, dried *in vacuo*, redissolved in 1 ml of water, and then counted for radioactivity of basic portion (C). The eluent (A) was dried *in vacuo* at 35°C, redissolved in 10 ml of water, and applied to a colum (1.37×6.8 cm) of Dowex 1 (×8; OH^- form; 200-400 mesh), and the column was eluted with a 50 ml of water at a flow rate of ml/min with 2 ml each fraction. Aliquots of every fraction were spotted on a filter paper, dried, and visualized by spraying of p-dimethylaminobenzaldehyde solution[10] (consisted of 2 g of p-dimethylaminobenzaldehyde, 100 ml of isopropanol, and 1 ml of conc. H_2SO_4) for dedection of urea and monomethylurea. The fractions of urea were pooled, dried *in vacuo* at 35°C, and dissolved in 2 ml of 0.15 M monomethylurea (internal standard). Aliquot of the solution was counted for radioactivity of neutral portion (D).

The same Dowex 1 column was eluted with 100 ml of 1 N HCl at a flow rate of 2 ml/min. And the eluents were pooled, dried *in vacuo*, redissolved in 1 ml of water, and then counted for radioactivity of the acidic portion (E) (Table 2 and scheme I).

Identification of monomethylurea

Thin-layer chromatograpgy

0.2 ml of neutral portion (D) (2,800 dpm) was applied on an avicel thin-layer plate (0.25 mm thickness), dried at room temperature, and developed in $CHCl_3$-MeOH-H_2O, 7:5:1. After developing, the plate was dried and visualized with p-dimethylaminobenzaldehyde reagent for finding out areas of monomethylurea. The avicel of the plate was scrapped out into 1 cm width, put it in counting vials containing 1 ml of water, shaked for a while, and then counted for radioactivity of monomethylurea (Fig. 1).

Ion-exchange resin column chromatography

1 ml of the neutral portion (D) (14,000 dpm) was passed down a column (1.37×6.8 cm) of Dowex 50 (×8; H^+ form; 200-400 mesh) and washed with 20 ml of water. The column was eluted with a gradient formed with 0.04-0.4 N HCl (200 ml each) at a flow rate of 1 ml per min with 4 ml fraction. Aliquots of every other fraction were used for counting of radioactivity of N-monomethylurea with 10 ml of scintillant. For determination of N-monomethylurea and

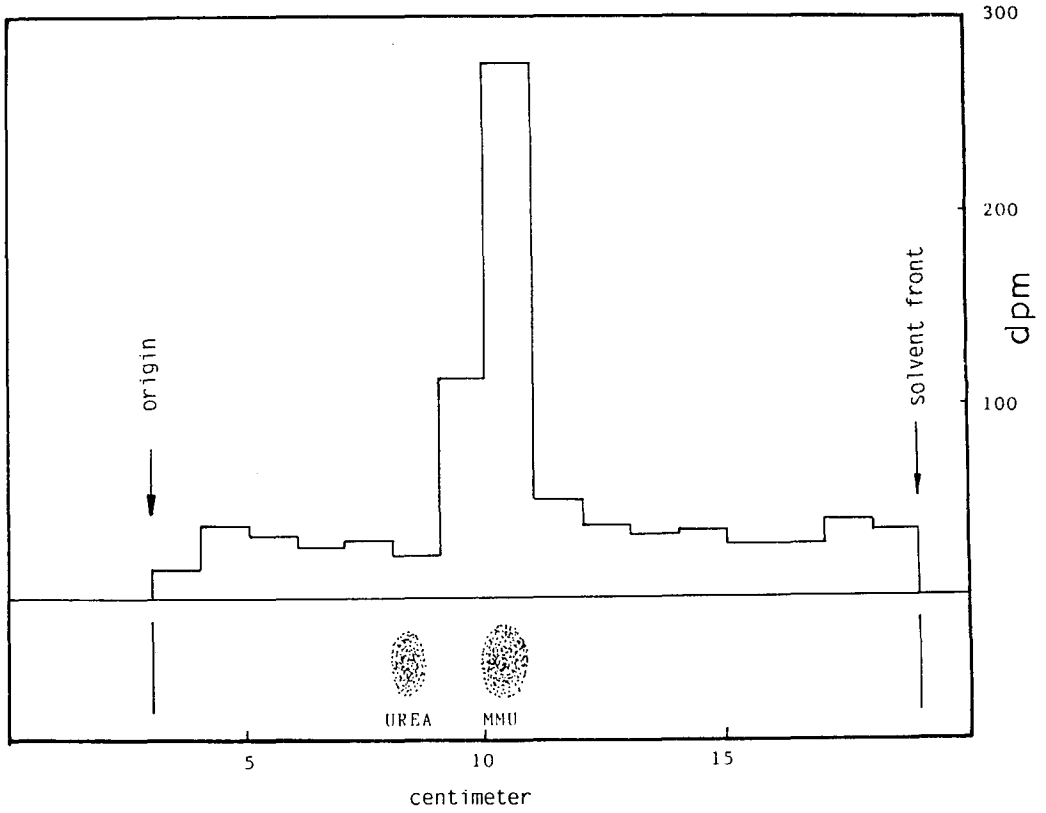


Fig. 1. Thin-layer chromatography of neutral portion.

The sample was placed on Avicel thin-layer plate for ascending chromatography in $CHCl_3$-MeOH-H_2O, 7:5:1. The avicel was scrapped out into 1 cm width and counted in scintillation counter. The standards (urea and N-monmethylurea) were detected with 2% solution of p-dimethylaminobenzaldehyde in isopropyl alcohol.

urea, 3 ml of reaction mixture contained aliquot of every other fraction, water, and 1.5 ml of the p-dimethylaminobenzaldehyde solution. The mixture was placed at room temperature for 10 min and read absorbance at 420 nm against reagent blank.

RESULTS AND DISCUSSION

Kakimoto and Akazawa[11] could not detected any trace of N^G-monomethyl-L-arginine in the human urine, but McDermott[5] determined that 0.4 μmol of the N^G-monomethyl-L-arginine is contained in 100 ml of human serum. An answer to the question of why N^G-monomethyl-L-arginine was missing in the urine while present in serum had come from the work of McDermott[5] in 1976. He injected N^G-monomethyl-L-arginine into rabbits and found that 0.14% of injected N^G-monomethyl-L-arginine was recovered unmetabolized in 24-hr urine sample and the five-carbon compounds, such as glutamic acid, proline, and ornithine were also found among the metabolic products excreted

Table 1. Radioactivity in Rat Urine after Oral Administration of N^G-monomethyl-L-arginine
N^G-monomethyl [^{14}C-methyl]-L-arginine hydrochloride (9×10^5 dpm) in 1ml of water was orally administrated; 24-hr urine samples were collected and radioactivity was determined.

Day	1	2	3	4	5	6	7	Total
Radioactivity (% of recovered)	57.2	4.35	2.02	1.03	0.61	0.59	0.56	66.36

in urine. The results clearly indicated that N^G-monomethyl-L-arginine was metabolized *in vivo*. After oral administration of ^{14}C-labelled N^G-mono[^{14}C-methyl]-L-arginine into rats, 66.3 % of the administered radioactivity had been recovered in the urine, and the radioactivity of the first 24-hr urine is 86.2% of the total of the recovered radioactivity (Table 1). By the ion-exchange resin column chromatography, distribution of radioactivity of the acidic, basic, and neutral portions and N^G-monomethyl-L-arginine are 33.3%, 40.2%, 12.5%, and 0.3%, respectively,

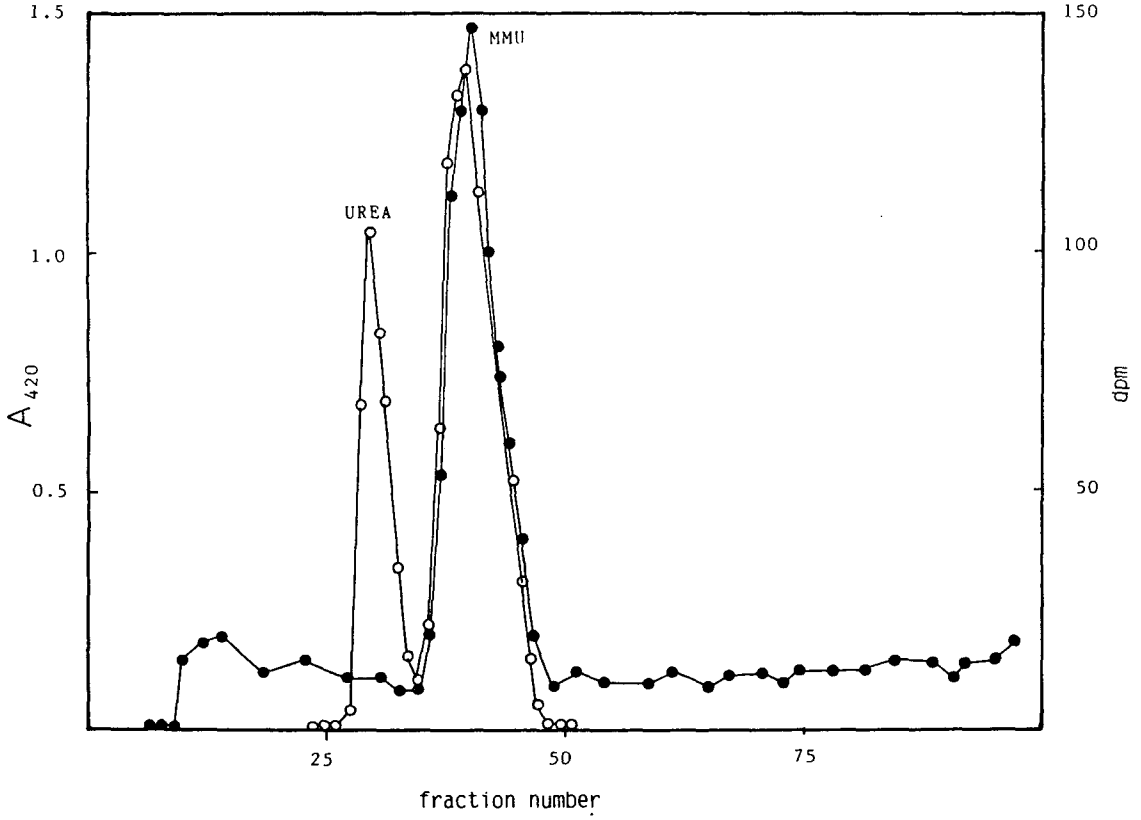


Fig. 2. The elution pattern of neural portion.

The sample was run on Dowex 50 (H^+ form) column (1.4×6.8 cm) and eluted with a gradient formed with 0.04-0.4 N HCl with 4 ml of fraction. Aliquots of the fraction were used for colorimetric determination of the standard (urea and N-monomethylurea) with 2% solution of p-dimethylaminobenzaldehyde in isopropyl alcohol, and for measuring radioactivity of N-monomethylurea. Radioactivity (●——●); absorbance (○——○).

Table 2. Distribution of Radioactivity on Column Chromatography

Portions	Radioactivity	
	dpm	%
Acidic [E]	74,800	34.0
Basic [C]	90,000	40.9
Neutral [D]	26,400	12.0
MMA [B]	720	0.3

The first 24-h urine sample (2.2×10^5 dpm) was applied on Dowex 50 (NH_4^+ form) and Dowex 1 (OH^- form), and eluted with 0.1 – 2.0 N NH_4OH, 6 N HCl, H_2O, and 1 N HCl for determinations of MMA, acidic, basic, and neutral portions, respectively.

as described in Table 2. 33.3% of the acidic portion indication the formation of acidic amino acid, glutamic acid, 40.2% of the basic portion which could be more alkaline than N^G-monomethyl-L-arginine (pI value, 10.55[8]) supports that the basic metabolite, such as N^G-monomethylagmatine, could be formed by enzytmatic decarboxylation of N^G-monomethyl-L-arginine in mammals.

The biological significance of the enzymatic formation of N^G-monomethylagmatine[12] is not obvious at present. The radioactivity of the N-monomethylurea formed was about 50% of the neutral portion (Table 2) and 6% of the administered radioactivity, which suggests that another metabolisms, such as demethylation, transamidination, and oxidation, could be formed enzymatically or nonenzymatically.

Thus, the present results show evidence of the endogeneous formation of N-monomethylurea which is a precursor of a potential carcinogen, 1-N-methyl-1-N-nitrosourea (MNU) (Fig. 1 and Fig. 2).

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