Effect of Pyridoxal on the Urinary Excretion of Nicotinamide Metabolites in Rats

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The effects of pyridoxal on the urinary excretion of nicotinamide metabolites such as *N*¹-methylnicotinamide (MNA), *N*¹-methyl-2-pyridone-5-carboxamide (2-Py), and *N*¹-methyl-4-pyridone-3-carboxamide (4-Py) were investigated. When a pharmacological amount of pyridoxal (500 μ mol/rat) was intraperitoneally injected into rats, the MNA excretion from 0 to 5 hr urine after the injection increased 5-fold, and concomitantly, the 2-Py excretion decreased by 1/2 and 4-Py excretion by 1/3. Therefore, the ratio of (2-Py + 4-Py) to MNA excretion was decreased greatly (1/15) by pyridoxal loading. These changes were gone by the 5-10 hr urine after the injection. In order to investigate these changes, the rats were killed 2 hr after the pyridoxal injection, and the activities of liver nicotinamide methyltransferase, 2-Py-forming MNA oxidase, and 4-Py-forming MNA oxidase were measured. Nicotinamide methyltransferase activity was increased slightly by the pyridoxal loading, but, 2-Py-forming MNA oxidase and 4-Py-forming MNA oxidase activity decreased greatly. Therefore, it was found that the increase in the urinary excretion of MNA and the concomitant decrease in the urinary excretion of 2-Py and 4-Py might be due to the inhibition of 2-Py-forming MNA oxidase and 4-Py-forming MNA oxidase activities by the pyridoxal loading.

Liver aldehyde oxidase was found in 1940 by Gordon *et al.*¹⁾ and Knox and Grossman²⁾ discovered that purified quinine-oxidizing enzyme (aldehyde oxidase) of rabbit liver oxidized *N*¹-methylnicotinamide (MNA) to *N*¹-methyl-2-pyridone-5-carboxamide (2-Py) in 1946, which was the first report on 2-Py. *N*¹-Methyl-4-pyridone-3-carboxamide (4-Py) was identified as a major metabolite of nicotinic acid and nicotinamide by Chang and Johnson³⁾ in 1959. Quinn and Greengard⁴⁾ reported that 4-Py was synthesized from MNA as well as 2-Py and that, in rats, two separate enzymes might be involved in the formation of the two pyridones 2-Py and 4-Py in 1966. Later (1978), Ohkubo and Fujimura⁵⁾ demonstrated this probability by purification of the two enzymes (2-Py-forming MNA oxidase and 4-Py-forming MNA oxidase). In 1971, Stanulovic and Chaykin⁶⁾ reported that the two MNA oxidases can also catalyze the conversion of pyridoxal to 4-pyridoxic acid *in vitro* and found that the urinary excretion

of [¹⁴C]2-Py and [¹⁴C]4-Py (first 6 hr urine after [¹⁴C]MNA loading) was lower when rats were concomitantly injected with pyridoxal (400 μ mol/rat) and [¹⁴C]MNA (1 μ mol/rat) than when rats were injected with only [¹⁴C]MNA (1 μ mol/rat). However, it is not clear (1) how long the decreased 2-Py and 4-Py excretion due to pyridoxal loading continues, (2) when 2-Py and 4-Py excretion decreases, how MNA is, and (3) whether the activities of 2-Py-forming MNA oxidase and 4-Py-forming MNA oxidase are lowered by pyridoxal loading or not *in vivo*. We have already reported that nicotinamide⁷⁾ or MNA⁸⁾ given to rats greatly decreased the liver 2-Py- and 4-Py-forming MNA oxidase activities. This experiment was done to resolve the above questions.

Materials and Methods

Chemicals. Pyridoxal-HCl and nicotinamide were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). MNA chloride was obtained from Tokyo Kasei

Kogyo Co., Ltd. (Tokyo, Japan). 2-Py and 4-Py were synthesized by the methods of Pullman and Colowick⁹⁾ and Shibata *et al.*,¹⁰⁾ respectively. All other chemicals used were of the highest purity obtainable.

Animals and diet. Ten male rats of the Wistar strain (seven weeks old) were purchased from Clea Japan Inc. (Tokyo, Japan). The rats were immediately placed individually in metabolic cages (CT-10; from Clea Japan) and fed a 20% casein diet [vitamin-free milk casein, 20%; L-methionine, 0.2%; α -cornstarch, 45.8%; sucrose, 23%; corn oil, 5%; mineral mixture (Oriental's ratio),¹¹⁾ 5%; vitamin mixture (Oriental's ratio),¹¹⁾ 1%] for 79 days. The food and water were given during urine collection. The light-darkness schedule was 06:00–18:00 (light) and 18:00–06:00 (dark). The room temperature was $22 \pm 2^\circ\text{C}$ and the humidity about 60%. At 09:00 hr on the 57th day, 500 μmol of pyridoxal per rat dissolved in 1 ml of sterilized saline was intraperitoneally injected into rats (body weight; 341.0 ± 7.4 g, mean $\pm$ S.E.M., $n=5$). Urine samples were collected in flasks containing 1 ml of 1 M HCl for –24–0 hr (day before injection), 0–5 hr, 5–10 hr, and 10–24 hr, following pyridoxal injection and stored at -25°C until analyze. These rats were continuously housed, and at 09:00 hr on the 80th day, 500 μmol of pyridoxal per rat dissolved in 1 ml of sterilized saline, was again intraperitoneally injected into the rats (body weight; 350.8 ± 12.2 g, $n=5$). As a control, 1 ml of saline was injected into the same-aged rats (body weight; 379.4 ± 12.4 g, $n=5$). These ten rats were killed 2 hr after the injection. The livers were removed, 5 volumes of cold 50 mM potassium phosphate buffer (pH 7.0) was immediately added and the livers were homogenized with a Teflon-glass homogenizer. The resulting homogenates were di-

rectly used as enzyme sources.

Analyses. The urinary excretion of 2-Py,¹⁰⁾ 4-Py,¹⁰⁾ and MNA¹²⁾ was measured by high-performance liquid chromatography. The methods for measuring the activities of nicotinamide methyltransferase, 2-Py forming-MNA oxidase, and 4-Py-forming MNA oxidase were described in refs. 13, 14, and 14, respectively.

Statistical analysis. The significance of differences was determined by analysis of variance and evaluated by Bartlett's test and Duncan's new multiple range test.¹⁵⁾

Results and Discussion

Table I shows the course of urinary excretion of MNA, 2-Py and 4-Py, and Table II shows the ratios of 4-Py to 2-Py excretion and of (2-Py+4-Py) to MNA excretion following injection of a large amount of pyridoxal (500 μmol /rat). The most striking effect was observed in the 0–5 hr urine after the injection, in which the urinary excretion of MNA increased 5-fold and concomitantly, that of 2-Py and 4-Py decreased by 1/2 and 1/3, respectively, in the comparison with the values of day before the injection when values are compared in terms of hourly urine. So the ratio of (2-Py+4-Py) to MNA excretion was greatly decreased by 1/15 by a pharmacological dose of pyridoxal in the 0–5 hr urine. This

Table I. EFFECTS OF PYRIDOXAL ON THE URINARY EXCRETION OF MNA, 2-PY AND 4-PY

	MNA		2-Py		4-Py	
	nmol 24 hr	nmol hr	nmol 24 hr	nmol hr	nmol 24 hr	nmol hr
–24–0 hr	568 ± 88	23 ± 4^a	526 ± 47	22 ± 2^a	8049 ± 448	335 ± 19^a
	nmol 5 hr	nmol hr	nmol 5 hr	nmol hr	nmol 5 hr	nmol hr
0–5 hr	558 ± 29	111 ± 6^b	68 ± 9	13 ± 2^b	517 ± 10	104 ± 10^b
	nmol 5 hr	nmol hr	nmol 5 hr	nmol hr	nmol 5 hr	nmol hr
5–10 hr	110 ± 27	22 ± 5^a	95 ± 14	19 ± 3^a	1627 ± 286	325 ± 47^a
	nmol 14 hr	nmol hr	nmol 14 hr	nmol hr	nmol 14 hr	nmol hr
10–24 hr	337 ± 53	24 ± 4^a	322 ± 27	23 ± 4^a	4410 ± 313	315 ± 42^a

Values are means $\pm$ S.E.M. for 5 rats; values with different superscript letters in the same column (nmol/hr) are statistically significantly different at $p < 0.05$.

Table II. EFFECTS OF PYRIDOXAL ON THE RATIOS OF 4-Py TO 2-Py AND OF (2-Py+4-Py) TO MNA EXCRETION

	4-Py/2-Py	(2-Py+4-Py)/MNA
-24-0 hr	15.5 ± 0.6 ^a	16.1 ± 1.7 ^a
0-5 hr	7.8 ± 0.4 ^b	1.1 ± 0.1 ^b
5-10 hr	10.5 ± 0.7 ^c	17.8 ± 2.1 ^a
10-24 hr	13.1 ± 0.8 ^a	15.4 ± 2.5 ^a

Values are means ± S.E.M. for 5 rats; values with different superscript letters in the same column are statistically significantly different at $p < 0.05$.

Table III. EFFECTS OF PYRIDOXAL ON THE ACTIVITIES OF NICOTINAMIDE METHYLTRANSFERASE, 2-Py-FORMING MNA OXIDASE AND 4-Py-FORMING MNA OXIDASE

	Control	Pyridoxal
Nicotinamide methyltransferase ($\mu\text{mol/hr/g liver}$)	0.76 ± 0.18 (100%)	1.00 ± 0.09 (132%)
2-Py-forming MNA oxidase ($\mu\text{mol/hr/g liver}$)	0.48 ± 0.03 ^a (100%)	0.15 ± 0.05 ^b (31%)
4-Py-forming MNA oxidase ($\mu\text{mol/hr/g liver}$)	1.59 ± 0.11 ^a (100%)	0.43 ± 0.15 ^b (27%)

Values are means ± S.E.M. for 5 rats; values with different superscript letters in the same row are statistically significantly different at $p < 0.05$.

Figures in parentheses are percentages of control.

might be due to the findings that liver nicotinamide methyltransferase activity was increased slightly by the pyridoxal loading, but the 2-Py-forming MNA oxidase and the 4-Py-forming MNA oxidase activities decreased as shown in Table III. The decreased activities of 2-Py-forming MNA oxidase and 4-Py-forming MNA oxidase are considered to be inactivated during the course of catalysis *in vivo* (pyridoxal → pyridoxic acid), because inhibitor(s) were not included in the homogenate of the pyridoxal group. The increase in MNA excretion and the decreases in 2-Py and 4-Py excretion were not observed in the 5-10 hr and 10-24 hr urine. In this connection, the circadian rhythm of urinary excretion of MNA,

2-Py, and 4-Py is not significant.^{7,8)} Therefore, this means that the pyridoxal effect diminished very fast. When a pharmacological amount of nicotinamide was injected into rats, the ratio of (2-Py+4-Py) to MNA excretion was the lowest in the 10-24 hr urine after the injection and returned to normal on day 4.⁷⁾ Therefore, the decrease and recovery speed was very much faster for the pyridoxal administration than for the nicotinamide administration. The ratio of 4-Py to 2-Py excretion in the 0-5 hr urine decreased by a half (15.5 → 7.8) in comparison with the value of day before the injection when a pharmacological dose of pyridoxal was administered. This ratio returned to a normal level in the 10-24 hr urine. In the excess nicotinamide or MNA administration, the ratio of 4-Py to 2-Py greatly decreased; the lowest value was around 0.5 in the nicotinamide⁷⁾ (obtained for the 10-24 hr urine after the injection) or MNA⁸⁾ administration (obtained at the 0-3 hr, 3-6 hr, and 6-9 hr urine). Accordingly, in comparison with the "nicotinamide or MNA experiment," the effect of pyridoxal on the ratio of 4-Py to 2-Py excretion was slight. This might be due to the result shown in Table III. The activities of 2-Py-forming MNA oxidase and 4-Py-forming MNA oxidase were equally inhibited by a pharmacological dose of pyridoxal. In the "nicotinamide experiment," the 2-Py-forming MNA oxidase activity was 45% of normal and the 4-Py-forming MNA oxidase activity 7%.⁷⁾ In the "MNA experiment," the 2-Py-forming MNA oxidase activity was 59% of normal and the 4-Py-forming MNA oxidase activity 11%.⁸⁾ The 4-Py-forming MNA oxidase is more unstable than the 2-Py-forming MNA oxidase; that is, 4-Py-forming MNA oxidase is more inactivated during the course of catalysis than 2-Py-forming MNA oxidase.⁶⁾ However, the activities of 2-Py-forming MNA oxidase and 4-Py-forming MNA oxidase were inhibited to the same extent by a pharmacological dose of pyridoxal in this experiment. Therefore, more pyridoxal production might be catalyzed by 2-Py-forming MNA oxidase than 4-Py-forming MNA oxidase.

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