Effects of Dietary Pyrazinamide, an Antituberculosis Agent, on the Metabolism of Tryptophan to Niacin and of Tryptophan to Serotonin in Rats

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The effects of pyrazinamide on the metabolism of tryptophan to niacin and of tryptophan to serotonin were investigated to elucidate the mechanism for pyrazinamide action against tuberculosis. Weanling rats were fed with a diet containing 0.25% pyrazinamide for 61 days. Urine samples were periodically collected for measuring the tryptophan metabolites. The administration of pyrazinamide significantly increased the metabolites, 3-hydroxyanthranilic acid and beyond, especially quinolinic acid, nicotinamide, N'-methylnicotinamide, and N'-methyl-4-pyridone-3-carboxamide, and therefore significantly increased the conversion ratio of tryptophan to niacin and the blood NAD level. However, no difference in the upper metabolites of the tryptophan to niacin pathway such as anthranilic acid, kynurenic acid and xanthurenic acid was apparent between the two groups. No difference in the concentrations of tryptophan and serotonin in the blood were apparent either. It is suggested from these results that the action of pyrazinamide against tuberculosis is linked to the increase in turnover of NAD and to the increased content of NAD in the host cells.

Key words: pyrazinamide; tryptophan; nicotinamide; quinolinic acid; tuberculosis

Nam, a vitamin, is biosynthesized from the essential amino acid, Trp, in the human body, and its biosynthesis might be strictly controlled since over 500 kinds of enzymes need pyridine nucleotides as a coenzyme and substrate. Accordingly, elucidation of the mechanism regulating the supply of pyridine nucleotides is linked to that of homeostasis in cells.

It is known that human varieties of Mycobacterium tuberculosis accumulate large quantities of NiA in the culture medium, whereas other mycobacteria do not. A study by Kasárov and Moat has revealed that extracts prepared from a human strain of M. tuberculosis had very high levels of NAD glycohydrolase and Nam deamidase when compared with the bovine strains. Furthermore, they have shown that the NiA phosphoribosyltransferase level was extremely low or absent in M. tuberculosis. Therefore, the human strain of M. tuberculosis can rapidly degrade NAD into NiA, but cannot recycle NiA into NAD. NiA then accumulates extracellularly.

PYZ and INH are used as essential components of the modern short-course regimen for tuberculosis treatment. The administration of INH causes pelagra, a typical niacin-deficiency syndrome. We investigated the effects of INH on the metabolism of Trp to niacin. The results show that the activity of kynurenine aminotransferase was significantly inhibited by feeding a diet containing INH, and that the urinary excretion of XA, a side-reaction product of the conversion pathway of Trp to niacin, was below the limit of detection. PYZ is also known as an antituberculous agent and hyperuricemic agent. Nasu et al. have reported that the 14C-labeled QA formation from L-[benzene-14C]Trp was markedly increased by loading with PYZ, while the metabolites from QA such as Nam and MNA were not significantly increased. However, we have previously revealed that the formation of Nam and such metabolites as MNA, 2-Py, and 4-Py were significantly increased by the administration of PYZ. Our previous work has indicated that antituberculosis agents disturbed the metabolism of Trp to niacin. Accordingly, further studies on the effects of antituberculosis agents on Trp metabolism could help to elucidate the target sites for antituberculosis agents.

In our previous studies, we could not measure

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Abbreviations: PYZ, pyrazinamide; INH, isonicotinic acid hydrazide; Trp, L-tryptophan; NiA, nicotinic acid; NaMN, nicotinic acid mononucleotide; Nam, nicotinamide; MNA, N'-methylnicotinamide; 2-Py, N'-methyl-2-pyridone-5-carboxamide; 4-Py, N'-methyl-4-pyridone-5-carboxamide; AnA, anthranillic acid; KA, kynurenic acid; XA, xanthurenic acid; 3-HA, 3-hydroxyanthranilic acid; QA, quinolinic acid; ACMS, O-amino-β-carboxymuconate-ε-semialdehyde; AMS, O-aminoacetoacetate-ε-semialdehyde; ACMSDase, aminocarboxymuconate-semialdehyde decarboxylase; QFRtase, quinolinolate phosphoribosyltransferase
Materials and Methods

Chemicals. NAD$^+$ was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Vitamin-free milk casein, sucrose, L-methionine, Nam, Trp, and AnA were purchased from Wako Pure Chemical Industries (Osaka, Japan). Kynurenine sulfate, MNA chloride, XA, KA, and 3-HA were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). 2-Py and 4-Py were synthesized by the methods of Pullman and Colowick$^{14}$ and of Shibata et al.$^{13}$ respectively. The mineral and vitamin mixtures were obtained from Oriental Yeast Kogyo (Tokyo, Japan), all other chemicals used being of the highest purity available from commercial sources.

Animals and diets. The animal room was maintained at a temperature of around 22°C and at about 60% humidity with a 12-h light/12-h dark cycle. The body weight and food intake were measured daily at around 10:00 a.m., and food and water were renewed daily.

The care and treatment of the experimental animals conformed with the University of Shiga Prefecture guidelines for the ethical treatment of laboratory animals. Male rats of the Wistar strain (3 weeks old) were obtained from Clea Japan (Tokyo, Japan) and immediately placed in individual metabolic cages (CT-10; Clea Japan). To accustom the rats to these conditions, they were initially fed ad libitum for 7 days with an NiA-free, 20% casein diet (control diet; Table 1). They were then divided into two groups, and fed ad libitum for 61 days, one group with the NiA-free, 20% casein diet, and the other with the same diet +0.25% pyrazinamide (Table 1). Urine samples (10:00 a.m.-10:00 a.m.; 24-h urine) were periodically (day 32-33, day 39-40, day 46-47, day 53-54, and day 60-61; see Figs. 3, 4, 5, and 6) collected in amber bottles with 1 ml of 1 M HCl and stored at −25°C until needed. The rats were killed by decapitation af-

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Table 1. Composition of the Diets

<table>
<thead>
<tr>
<th></th>
<th>Control diet (NiA-free 20% casein diet) (%)</th>
<th>Test diet (control diet + 0.25% PYZ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin-free casein</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Gelatinized cornstarch</td>
<td>45.9</td>
<td>45.65</td>
</tr>
<tr>
<td>Sucrose</td>
<td>22.9</td>
<td>22.9</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mineral mixture*</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>vitamin mixture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NiA-free)**</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PYZ</td>
<td>0</td>
<td>0.25</td>
</tr>
</tbody>
</table>

* The compositions of the mineral and vitamin mixtures are those of Oriental Yeast Kogyo Co. (Tokyo).
** NiA-free means that nicotinic acid has been removed from the vitamin mixture.

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Fig. 1. Metabolic Pathway from Trp.
Dotted line: mammals cannot conventionally convert Nam into NiA.
ter the last urine samples had been collected, and a 10-μl sample of blood was taken from the carotid artery of each and treated as described in the literature for measuring NAD (NAD⁺ + NADH) and NADP (NADP⁺ + NADPH). The liver of each animal was removed, and a portion (approximately 1 g) was immediately homogenized with a Teflon-glass homogenizer in five volumes of a cold 50 mM KH₂PO₄-K₂HPO₄ buffer (pH 7.0). One part of each homogenate was centrifuged at 105,000 × g for 20 min to measure the activities of 3-HA oxygenase, QPR-Tase, and ACMSDase.

**Analyses.** The liver activities of 3-HA oxygenase (EC 1.13.11.6), QPR-Tase (EC 2.4.2.19), and ACMSDase (EC 4.1.1.45) were measured as described in the literature. The contents of Nam, 2-Py, and 4-Py in the urine were simultaneously measured by the HPLC method of Shibata et al., while the content of MNA in the urine was measured by the HPLC method of Shibata. The contents of KA, XA, 3-HA and AnA in the urine were measured by the HPLC method, while the contents of NAD (NAD⁺ + NADH) and NADP (NADP⁺ + NADPH) were measured by the colorimetric method of Shibata and Murata, and Shibata and Tanaka, respectively.

**Results**

**Effect of PYZ on the body weight gain and food intake**

In a previous study, the amount of added PYZ was 0.1%, and the experimental period was 31 days. In the present experiment, to manifest the effect of PYZ on the metabolism of Trp, the added amount of PYZ was raised to 0.25% and the experimental period was increased 2-fold; weaning rats were therefore fed with a diet containing 0.25% PYZ for 61 days. No difference in the body weight gain and food intake was apparent between the control and PYZ groups, as shown in Fig. 2. No macroscopic adverse effect of PYZ was seen during the experiment.

**Effect of PYZ on the conversion ratio of Trp to niacin**

We checked whether the conversion ratio of Trp to niacin increased or not under the present conditions. The conversion ratio of Trp to niacin is usually calculated by the equation \[(\text{sum of Nam, MNA, 2-Py, and 4-Py}) / \text{Trp intake} \] during urine collection. However, in the present experiment, 2-Py and PYR were co-eluted with our method, so the urinary excretion of 2-Py in Fig. 3-C includes PYZ voided into the urine. Accordingly, in this paper, the conversion ratio of Trp to niacin was calculated by comparing the sum of Nam, MNA, and 4-Py with the Trp intake.

PYZ administration markedly increased the urinary excretion of Nam, MNA, 2-Py (the value contains PYZ only in the PYZ group) and 4-Py, as shown in Fig. 3. In particular, the urinary excretion of MNA was 50-fold higher by the PYZ group than by the control group. In contrast, the increased urinary excretion of 4-Py was only 6-fold. The calculated conversion ratio is shown in Fig. 4, the ratio being around 7.5-fold higher in the PYZ group than in the control group.

**Effect of PYZ on the urinary excretion of QA.**

Nasu et al. have reported that the administration of PYZ caused an inhibition of ACMSDase activity and showed an increase in ¹⁴C-QA from ¹⁴C-Trp. Although QA is a key metabolite of the Trp-niacin pathway, the practical measurement of QA was not reported. The increase in QA excretion during the feeding with a PYZ diet had not previously been evaluated. As is shown in Fig. 5, QA formation was increased

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**Fig. 2.** Comparison of the Body Weight Gain (A) and Food Intake (B) of Rats Fed with the Control Diet (NiA-free, 20% Casein Diet) and Test Diet (NiA-free, 20% Casein Containing 0.25% PYZ).

○, control diet; ●, test diet. Each point is the mean ± SEM (n = 5).
10-fold by the PYZ loading, suggesting the inhibition of ACMSDase activity by PYZ in vivo as reported by Nasu et al.10

**Effect of PYZ on the urinary excretion of upper metabolites of Trp to niacin**

The effects of PYZ on the urinary excretion of AnA, KA, XA, and 3-HA was investigated (Fig. 6). While the urinary excretion of AnA, XA, and KA was not affected by feeding the PYZ diet, the urinary excretion of 3-HA was increased 3.5-fold. This result indicates that PYZ increased the formation of 3-HA.

**Effect of PYZ on some enzyme activities concerned with the conversion of Trp to niacin**

It is possible from the results of the urinary excretion of the Trp metabolites that PYZ affected the formation of 3-HA and/or QA. Thus, the activities of 3-HA oxygenase, ACMSDase, and QPRTase in the liver were investigated. The activity of ACMSDase was no lower in the PYZ group than in the control group as shown in Table 2, and the activities of 3-HA
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Oxygenase and QPRTase were similarly not affected by the PYZ loading (Table 2).

Effect of PYZ on the NAD and NADP contents in blood
The blood NAD content can be used as an index of systemic niacin nutrition.\(^{10}\) PYZ loading caused an increase in the blood NAD content, as shown in Table 3, although the NADP content did not increase (Table 3).

Effect of PYZ on the Trp and serotonin contents in blood
PYZ loading of rats did not affect the contents of Trp and serotonin in the blood as shown in Table 3.

Discussion

Human \textit{M. tuberculosis} has a unique biosynthetic pathway for NAD; the strain has very high levels of the NAD-degrading enzyme and Nam deamidase, but an extremely low level of NiA phosphoribosyltransferase.\(^{23}\) Therefore, the microorganism can rapidly degrade NAD to NiA, but cannot recycle NiA to NAD,\(^ {2}\) but...
Table 2. Effect of PYZ on the Enzymes Concerned with QA Formation and Degradation

<table>
<thead>
<tr>
<th></th>
<th>Control diet (NIA-free 20% casein diet)</th>
<th>Test diet (control diet + 0.25% PYZ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-HA oxygenase</td>
<td>687 ± 70</td>
<td>722 ± 24</td>
</tr>
<tr>
<td>ACMSDase</td>
<td>3.06 ± 0.38</td>
<td>3.81 ± 0.64</td>
</tr>
<tr>
<td>QPRTase</td>
<td>0.80 ± 0.03</td>
<td>0.82 ± 0.03</td>
</tr>
</tbody>
</table>

Each value is expressed as nmol/h/g of liver and is the mean ± SEM for five rats.

1 The reaction mixture (3 ml in a cuvette) containing 0.1 ml of 3.3 mM 3-HA (dissolved in a 50 mM Tris-acetate buffer at pH 8.0), 1 ml of a 0.2 M Tris-acetate buffer at pH 8.0, and 1.8 ml of water was incubated for 5 min at 25°C. The reaction was started by the injection of 20 μl of an enzyme source, and the rate of the reaction was calculated at 360 mm from the linear portion of the optical density versus time curve.

2 The reaction mixture (3 ml in a cuvette) containing 20 μl of 3.3 mM 3-HA (dissolved in a 50 mM Tris-acetate buffer at pH 8.0), 1 ml of a 0.2 M Tris-acetate buffer at pH 8.0, and 1.8 ml of water was incubated for 5 min at 25°C. The ACMS substrate was made by the addition of an excess quantity of a purified preparation of 3-HA oxygenase (approximately 0.4 mg of protein per 50 μl). After the formation of ACMS was complete, as judged by its absorbance at 360 nm, 0.1 ml of an enzyme source was added. The absorbance at 360 nm was followed for 10 min against a blank incubation that contained all the ingredients except the substrate. These data were corrected for the spontaneous decrease in absorbance due to the formation of QA.

3 The incubation medium (a final volume of 500 μl) contained 50 μl of a 500 mM KH2PO4-K2HPO4 buffer (pH 7.0), 50 μl of 10 mM QA, 50 μl of 10 mM PRPP, 10 μl of 100 mM MgCl2, 260 μl of H2O, and 50 μl of an enzyme source. The reaction was started by the addition of the enzyme source, and the incubation was carried out at 37°C for 1 h. The reaction tube was placed in a boiling water bath for 5 min and then centrifuged to separate the supernatant from the enzyme source. The supernatant was filtered through a 0.45-μm microfilter, and the filtrate (20 μl) was injected into the HPLC system.

Table 3. Effect of PYZ on the NAD, NADP, Trp, and Serotonin Contents in Blood

<table>
<thead>
<tr>
<th></th>
<th>Control diet (NIA-free 20% casein diet)</th>
<th>Test diet (control diet + 0.25% PYZ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD</td>
<td>92.2 ± 5.4</td>
<td>114.4 ± 5.5*</td>
</tr>
<tr>
<td>NADP</td>
<td>13.7 ± 1.4</td>
<td>14.4 ± 0.6</td>
</tr>
<tr>
<td>Trp</td>
<td>61.8 ± 2.2</td>
<td>60.6 ± 1.6</td>
</tr>
<tr>
<td>Serotonin</td>
<td>11.1 ± 0.6</td>
<td>11.5 ± 0.1</td>
</tr>
</tbody>
</table>

Each value is expressed as nmol/ml of whole blood and is the mean ± SEM for five rats.

* Significant difference from the control group at p<0.05 by the Student t-test.

only when NIA is exogenously supplied; mammals cannot convert NIA into NIA since the Km value of Nam deamidase is around 1 mol/L.25

Antituberculosis agents such as INH and PYZ disturb the metabolism of Trp; for example, the administration of INH causes pellagra18 and an extremely low excretion of XA into the urine,3 while PYZ causes an increase in the formation of Nam.11,12 In the present experiment, the administration of PYZ markedly increased the formation of Nam and its metabolites (Fig. 3), and the conversion ratio of Trp to Niacin, therefore, was significantly increased (Fig. 4). Although it has not previously been known how the urinary excretion of QA would be increased by the PYZ intake, the present experiment has clearly revealed that it was increased about 10-fold by the PYZ loading (Fig. 5). QA was non-enzymatically and spontaneously formed from ACMS, ACMS being formed from 3-HA by 3-HA oxygenase, which was not affected by PYZ loading (Table 2). The activity of 3-HA oxygenase was much higher than the activities of ACMSDase and QPRTase (Table 2); therefore, the change in its activity did not affect the formation of ACMS. This result indicates that the reaction of ACMS→AMS was inhibited by PYZ loading; namely, it is probable that the activity of ACMSDase was inhibited and, as a result, the formation of QA increased (see Fig. 1). The level of liver ACMSDase was measured by using the supernatant of the 5-fold-diluted liver homogenate. However, the activity of ACMSDase was no lower in the PYZ group than in the control group (Table 2). Nasu et al.10 have reported that the liver ACMSDase activity decreased when rats were intraperitoneally injected with PYZ (300 mg/kg of body weight) and killed 4 h after the injection (they used the supernatant of the 4-fold-diluted liver homogenate as an enzyme source). In the present experiment, the rats were fed a diet incorporating 0.25% PYZ and, therefore, the average PYZ intake was 190 mg/kg of body weight/day (15 g (food intake/day) x (0.25/100) (the content of PYZ in the diet) x 0.2 g (the body weight of a rat)). The contradictory phenomenon of the increased QA formation and the non-decreased ACMSDase activity in the present experiment might be a result of the concentration of an inhibitor in the enzyme source.

The administration of a large amount of QA to rats has resulted in little increase in the formation of Nam and its metabolites,6,26 namely, dietary QA has very low niacin activity. QA can be absorbed into the body because a large amount of QA itself is excreted into the urine.26 Nevertheless, QA cannot be efficiently utilized for the biosynthesis of NAD. There must be a little QA transported from the blood into the liver cells where QA is converted to NaMN by QPRTase and then synthesized to NAD. On the other hand, endogenous QA in the liver cells is efficiently converted into NaMN. Thus, the contents of NAD and its metabolites are markedly increased. For such upper metabolites of Trp as AnA, KA, and XA, the excretion of each into the urine was almost the same between the control and PYZ groups (Fig. 6). The excretion of 3-HA, another upper metabolite, was higher in the PYZ group than in the control group (Fig. 6). The mechanism for the increased 3-HA formation by PYZ cannot be clearly explained, but it is possible that PYZ induced the cytochrome P-450 system which might catalyze the hydroxylation reaction of AnA, namely AnA→3-
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HA. Although this reaction is not the main pathway under normal conditions, it is probable that the reaction would be significantly increased by PYZ loading to increase the formation of 3-HA. Although there was no higher activity of QPRase in the PYZ group than in the control group, NAD and such metabolites as Nam, MNA, 2-Py and 4-Py were significantly increased. This finding indicates that the rate-limiting factor in the reaction of QA→MNA is an abundant supply of QA, and not QPRase itself, since there are no reports on activators of QPRase.

The neuroexcitatory endogenous Trp metabolite, QA, has caused neuronal lesions after intrastriatal and intrahippocampal injections in the rat. In the present experiment, the QA formation was much higher in the PYZ group than in the control group, although the food intake and body weight gain were almost the same between both groups and no aberrational behavior in the PYZ group was apparent. Therefore, QA cannot penetrate the blood brain barrier.

Pyrazinamide did not affect the metabolism of Trp to serotonin and the blood content of Trp. It is suggested from these results that the action of pyrazinamide against tuberculosis is linked to increases in the turnover of NAD and in the content of NAD through the inhibition of ACMSDase and to the increased reaction of AnA→3-HA in the host cells.

References


