Isolation and Identification of \( N^1 \)-Methylnicotinic Acid (Trigonelline) from Rat Urine

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(Received September 14, 1984)

Summary A urinary metabolite of nicotinic acid, single- and double-labeled \( N^1 \)-methylnicotinic acid (trigonelline: Tg) were isolated as urinary excretion products of rats following portal vein injection of \([\text{carboxyl-}^{14}\text{C}]\text{nicotinic acid (}^{14}\text{C-NiA})\) and/or \( S\)-adenosyl-\( L\)-[methyl-\( ^3\text{H}\)]methionine (\( ^3\text{H-SAM}\)). The labeled compounds produced were identified as Tg by comparison of ultraviolet absorption pattern and chromatographic behavior with those of the authentic compound, and by cocrystallization with the authentic carrier. It was demonstrated that enzymatic synthesis of double-labeled Tg is accomplished by reaction between \( ^{14}\text{C-NiA} \) and \( ^3\text{H-SAM} \), and the enzymatic product was identified using the above methods. Approximately 1.5\% respectively of the radioactivity of \( ^{14}\text{C-NiA} \) and \( ^3\text{H-SAM} \) was incorporated into Tg by enzymatic reaction. Furthermore, Tg from normal rat urine was isolated by ion-exchange chromatographic procedures and was crystallized from ethyl alcohol. The isolated compound was identified as Tg by comparison of ultraviolet spectra, infrared spectra, gas-mass spectra, elemental analysis, melting point and thin layer chromatogram behavior with those of the authentic compound. From these results, it was clearly demonstrated that Tg was one of the metabolites of nicotinic acid in the rat.

Key Words \( N^1 \)-methylnicotinic acid, trigonelline, thin-layer chromatography, nicotinic acid metabolism

A number of compounds have been described as metabolites of nicotinic acid or nicotinamide in mammals. These are nicotinic acid (1), nicotinuric acid (2), nicotinamide (2), Tg (2), \( N^1 \)-methylnicotinamide (3-6), \( N^1 \)-methyl-2-pyridone-5-carboxamide (7), \( N^1 \)-methyl-4-pyridone-3-carboxamide (8), nicotinamide-N-oxide (9), 6-hydroxynicotinic acid (10) and 6-hydroxynicotinamide (10). In earlier studies (1, 2) the metabolites were isolated from the urine of animals and identified...
by chemical analysis. Since the physiological importance of nicotinamide was established, large series of analysis have been carried out by routine assay methods. Most of them were based on the König reaction. When N1-methylnicotinamide was found to be a metabolite, this compound was assayed by the alkaline acetone condensation method (11, 12). It soon became evident that most of metabolite previously described as Tg was in fact N1-methylnicotinamide. Huff and Perlzweig (13), Ellinger and Abder Kader (14), Perlzweig et al. (15), Leifer et al. (16) were not able to demonstrate the presence of Tg as a metabolite of nicotinic acid in normal rats and mice. On the other hand, Mason and Kodicek (17) reported that the presence of Tg in rat urine is partly due to metabolism of dietary niacin. Tarr showed that Tg is excreted in normal human urine (18). In our previous experiment,2 the radioactivity of [carbonyl-14C]nicotinamide injected into the portal vein of rat was detected in the Tg fraction on the cellulose thin-layer chromatogram of urine and the labeled compound was tentatively identified as Tg by its ultraviolet spectrum and chromatographic behavior.

In this paper, single- and double-labeled Tg were identified from rat urine following portal vein injection of 14C-NiA and/or 3H-SAM. It was demonstrated that Tg synthesis by rat liver enzyme proceeds in a manner analogous to the formation of N1-methylnicotinamide. This compound was also isolated from normal rat urine and crystallized from ethyl alcohol. The crystallized compound was identified as Tg by comparison of ultraviolet spectra, infrared spectra, gas-mass spectrometry, elemental analysis, behavior on TLC and melting point with those of the authentic compound.

MATERIALS AND METHODS

**Materials.** [Carboxyl-14C]nicotinic acid (50.7 mCi/mmol) was purchased from the Radiochemical Centre (Amersham, UK), S-adenosyl-L-[methyl-3H]methionine (67 Ci/mmol) from New England Nuclear (Boston, Mass., USA), S-adenosyl-L-methionine from Boehringer Mannheim (Darmstadt, FRG), N1-methylnicotinic acid hydrochloride (Tg) from Sigma Chemical Co. (St. Louis, Mo., USA), Avicel® SF TLC cellulose plate from Funakoshi Pharmaceutical Co., Ltd. (Tokyo, Japan), SP-Sephadex C-25 from Pharmacia Co. (Uppsala, Sweden), and Dowex 1-X8 (200–400 mesh) and Dowex 50W-X12 (200–400 mesh) from Dow Chemical Co. (Middland, Mich., USA). Developing solvents of guaranteed reagent grade were obtained commercially. Male Wistar rats were obtained from Clea Japan, Inc. (Tokyo, Japan).

**Methods**

1) **Identification of single- and double-labeled Tg from rat urine in vivo.** Five

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2 Unpublished data were presented before the X International Congress of Nutrition at Kyoto, August, 1975 and the XII International Congress of Nutrition at San Diego, August, 1981.
male Wistar-strain rats, each weighing from 250 to 300 g were maintained on 18% casein diet (19) for 2 weeks and \(^{14}\)C-NiA (6 μCi, 0.12 μmol) or \(^{14}\)C-NiA (10 μCi, 0.20 μmol) and \(^3\)H-SAM (10 μCi, 0.15 nmol) were injected into the portal vein under pentobarbital sodium anesthesia, and urine was then immediately collected directly via a tube from the bladder for 4 h. All urine samples were stored at \(-20^\circ C\) until use. The urinary metabolites were separated using six multiple development (ascending) for four solvent systems. After detecting the unidentified compound with a model UV SL-50 mineral lamp, the unidentified compound with cellulose on the plate was scraped off and then extracted with water. The extract was pooled and evaporated to dryness under reduced pressure in a 60°C water bath. For cocrystallization, the authentic compound was added to the residue, and the compound was recrystallized from ethyl alcohol. The labeled Tg-like fraction was identified as Tg by cocrystallization with the authentic carrier. Radioactivity was determined with a Aloka model LSC-653R liquid scintillation spectrometer. The extracted solution was identified as Tg by the determination of \(R_f\) value and UV absorption spectrum. \(R_f\) value was verified by a single development with three solvent systems \([A: n\text{-propyl alcohol}\text{-conc. ammonia\text{-methyl alcohol}} (5:2:1, v/v/v), B: n\text{-butyl alcohol\text{-acetic acid\text{-water}} (6:2:2, v/v/v) and C: n\text{-propyl alcohol\text{-3\% aqueous ammonia}} (5:4, v/v)].\) Ultraviolet spectra were measured with a Shimadzu UV-300.

2) Enzymatic synthesis of double-labeled Tg by rat liver enzyme in vivo. The crude enzyme preparation was prepared from normal liver using part of Cantoni's method (20) on nicotinamide methyltransferase. The enzyme was prepared by heat treatment at 50°C and ammonium sulfate fractionation between 30 to 60% saturation. Specific activity of this enzyme on \(N^1\)-methylnicotinamide formation was about 19.04 nmol/mg protein/h. Protein was determined by the \(A_{280}: A_{260}\) method. The incubation mixture (20) was modified as follows: 1 μmol of nicotinic acid, 1 μmol of \(S\)-adenosyl-L-methionine, 25 μmol of acetate buffer (pH 5.0), 100 μl of crude enzyme (10 mg protein/ml), 1 μCi (0.02 μmol) of \(^{14}\)C-NiA and 2 μCi (0.03 nmol) of \(^3\)H-SAM in a total volume of 500 μl. After incubation for 120 min at 37°C, tubes were cooled and 1 ml of 10% trichloroacetic acid was added to the incubation mixtures. The precipitated protein was removed by centrifugation, and the supernatant was neutralized with 1N NaOH. The double-labeled Tg-like compound produced enzymatically was identified by the same procedures as described in Method 1.

3) Isolation and identification of Tg from normal rat urine. Ten male Wistar strain rats, each weighing 90 to 100 g were maintained on 18% casein diet for 2 weeks. The rats were placed in metabolism cages, and daily urine samples were collected under acetate acid and pooled for 10 days. Urine was passed through a Dowex 1-X8 (Cl\(^-\)) column (50 × 150 mm) and the column was washed with water. The effluent and washing were combined and the solution was then placed on a column of Dowex 50W-X12 (25 × 300 mm) in the H\(^+\) form. The column was washed with water and eluted with linear hydrochloric acid gradient (0.7–2.0 N), and the
eluate was evaporated to dryness under reduced pressure. The residue was dissolved in water and the solution was then applied to a column of SP-Sephadex C-25 (25 × 300 mm) and eluted with water. The Tg fractions were pooled and the pooled solution was evaporated to dryness. Finally, the isolated compound was crystalized from ethyl alcohol, and identified by comparison of ultraviolet spectra, infrared spectra, gas-mass spectrometry, elemental analysis, chromatographic behavior and melting point with those of the authentic compound.

RESULTS

1) Identification of single- and double-labeled Tg from rat urine in vivo

The first evidence for a hitherto unidentified metabolite of nicotinic acid was obtained during attempts to develop a thin-layer chromatographic procedure for the variation of excretion pattern of nicotinic acid metabolites. When urine of rats with 14C-NiA injected into the portal vein was chromatographed with six multiple developments for four solvent systems, many labeled compounds were found separated on the plate. The labeled Tg-like fraction at Rf value 0.39 corresponding to the authentic compound was extracted as mentioned in MATERIALS AND METHODS, and verified as Tg by cocrystallization with the authentic compound. As

Table 1. Cocrystallization of urinary radioactive trigonelline-like compound with authentic carrier.
The single (A) and double (B) labeled trigonelline-like fractions which were separated by six multiple developments with four solvent systems (Rf, 0.39) were extracted with water from cellulose plate, and the extracts were evaporated to dryness; 500 mg of authentic compound was added to each residue and the compounds were recrystallized seven times (A) and five times (B) from ethyl alcohol.

| (A)          | Specific activity following recrystallization |
|--------------|---------------------------------------------|
| 14C-Trigonelline (dpm/μmol) | 1st | 2nd | 3rd | 4th | 5th | 6th | 7th |
|              | 18.13 | 18.05 | 13.81 | 15.15 | 12.12 | 14.81 | 13.91 |

| (B)          | Specific activity and ratio of 3H/14C following recrystallization |
|--------------|------------------------------------------------------------------|
| 14C-3H-Trigonelline (dpm/μmol) | 1st | 2nd | 3rd | 4th | 5th |
| 14C          | 0.456 | 0.319 | 0.366 | 0.279 | 0.280 |
| 3H           | 3.130 | 1.831 | 1.122 | 0.937 | 1.164 |
| Ratio of 3H/14C | 7.2  | 5.7  | 3.1  | 3.4  | 4.2  |

High specific activity of (A) was caused by the radioactive trigonelline-like compound that was extracted from a large number of plates.

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Table 2. *R*<sub>f</sub> values of radioactive trigonelline-like compound on the cellulose plate, using various solvent systems.

Solvent systems A, B and C are described in Materials and Methods.

| Solvent system | Solvent system | Solvent system |
|----------------|----------------|----------------|
| A              | B              | C              |
| Authentic trigonelline | 0.32 | 0.20 | 0.51 |
| 14C-Trigonelline     | 0.32 | 0.21 | 0.52 |
| 14C-3H-Trigonelline  | 0.32 | 0.21 | 0.52 |

Shown in Table 1A, the specific activity of the single-labeled compound remained essentially constant after the 5th recrystallization. To establish further the identity of the Tg compound, 14C-NiA and 3H-SAM were respectively injected into the portal veins of 2 rats. Double-labeled Tg-like compound was verified as Tg by the same procedures. As shown in Table 1B, the specific activity of the double-labeled compound remained essentially constant at 14C and 3H, and the ratio of 14C to 3H also remained constant after the 3rd recrystallization. *R*<sub>f</sub> values of single- and double-labeled extracted compounds coincided with the *R*<sub>f</sub> value of authentic Tg as shown in Table 2. The ultraviolet absorption spectra of these two compounds in water exhibited a maximum at 265 nm, a minimum at 244 nm and a shoulder in the neighborhood of 268 to 270 nm which were almost identical with those of authentic Tg.

From these data, single- and double-labeled Tg were found to be excreted in rat urine.

2) **Enzymatic synthesis of double-labeled Tg by rat liver enzyme in vitro**

The finding that 14C of 14C-NiA and 3H of 3H-SAM were incorporated into Tg excreted in urine would suggest a conversion of labeled materials into labeled Tg by rat liver enzyme. In order to verify this, the synthesis of Tg in vitro by rat liver enzyme preparation containing nicotinamide methyltransferase was attempted. As described in Method 2, after incubation of the reaction mixture containing 14C-NiA and 3H-SAM, double-labeled Tg compound as reaction product was extracted. The results of recrystallization of the Tg-like compound are shown in Table 3. The specific activity of compound remained essentially constant at 14C and 3H, and the ratio of 14C to 3H also remained constant after 7th recrystallization. *R*<sub>f</sub> values of extracted Tg-like compound were almost identical with those of authentic Tg as shown in Table 4. Also, the ultraviolet absorption spectrum of extracted Tg-like compound was almost identical with that of authentic Tg. And the neutralized supernatant of the reaction mixture was chromatographed without authentic Tg by a single development for three solvent systems (A, B, C). The radioactive Tg fraction with cellulose on the plate was scraped off and the radioactivity determined. As shown in Table 4, the percentage of radioactivity of double-labeled Tg of the total activity on the plate was approximately 1.5% in both 14C and 3H. Therefore, it was suggested that the labeled Tg was produced by rat liver enzyme.
Table 3. Cocrystallization of enzymatically synthesized radioactive trigonelline-like compound with authentic carrier.
After incubation of reaction mixture, neutralized solution was applied to the cellulose plate. The double-labeled trigonelline-like fraction which was separated by single development with a solvent system A (described in Materials and Methods 1) was extracted with water from cellulose plate. The extract was evaporated to dryness; 500 mg of authentic compound was added, and the compound was recrystallized eight times from ethyl alcohol.

| Specific activity and ratio of $^3$H/$^{14}$C following recrystallization |
|-----------------------------|-----------------|-----------------|-------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                           | 1st             | 2nd             | 3rd | 4th | 5th | 6th | 7th | 8th |
| $^{14}$C-$^3$H-Trigonelline |                 |                 |     |     |     |     |     |     |
| (dpm/μmol)                 | $^{14}$C        | $^{3}$H         |     |     |     |     |     |     |
| 14C                        | 3.18            | 1.56            | 0.43 | 0.41| 0.36| 0.25| 0.27| 0.30| 0.23| 0.23 |
| Ratio of $^3$H/$^{14}$C    |                 |                 |     |     |     |     |     |     |

Table 4. $R_f$ values of radioactive trigonelline and incorporation of radioactive materials into trigonelline formed by rat liver enzyme.
A 25 μl portion of neutralized solution was applied to the cellulose plate. Plates were developed with three solvent systems (described in Materials and Methods 1). Each trigonelline fraction located as trigonelline was scraped off, and radioactivities were measured. Total activities of $^{14}$C and $^3$H on the plate were 27,657 and 16,843 dpm, respectively.

| $R_f$ value of authentic trigonelline | Solvent system A | Solvent system B | Solvent system C |
|-------------------------------------|------------------|------------------|------------------|
|                                     | 0.32             | 0.21             | 0.52             |
| $R_f$ value of synthesized trigonelline | 0.32             | 0.21             | 0.52             |

| Radioactive trigonelline (dpm) | Solvent system A | Solvent system B | Solvent system C |
|--------------------------------|------------------|------------------|------------------|
| $^{14}$C                        | 441 (1.59)       | 420 (1.52)       | 382 (1.38)       |
| $^3$H                           | 236 (1.70)       | 242 (1.44)       | 260 (1.54)       |

*Figures in parentheses are expressed as percentage of radioactive trigonelline for the total activity on the plate.

These results clearly demonstrate that the double-labeled Tg was formed by rat liver enzyme.

3) Isolation and identification of Tg from normal rat urine
Urine sample (1/5 of pooled urine) was passed through a Dowex 1-X8 (Cl−) column and the column was fully washed with water. The effluent and washing were combined and the solution was evaporated to dryness. The above procedure was repeated 5 times. The residue was dissolved in 500 ml of water. Figure 1 shows a
Fig. 1. Isolation of the unknown metabolite by ion-exchange chromatography. (A) Dowex 50W-X12 chromatography: Fifty ml of the dissolved solution was applied to a column of Dowex 50W-X12 in the H⁺ form (25 × 300 mm). The column was washed with 500 ml of water, 300 ml of 0.5 N HCl and 500 ml of water, and the washing solution was then discarded. The column was eluted with the linear HCl gradient solution (1,000 ml of 0.7 N HCl to 1,000 ml of 2 N HCl) and 20 ml fractions were collected. The absorbance at 260 nm was measured with an Ohtake Auto UV-2800. Fractions 48 to 85 were pooled and evaporated to dryness under reduced pressure. This treatment was repeated 10 times. The residue fraction contained N¹-methylnicotinamide, N¹-methyl-4-pyridone-3-carboxamide, trigonelline-like compound and others. This residue was dissolved with 50 ml of water. (B) SP-Sephadex C-25 chromatography: A 3 ml portion of solution obtained in (A) was applied to a SP-Sephadex C-25 column (25 × 300 mm) and eluted with water, and 10 ml fractions were collected. The absorbance at 260 nm was measured as above. Peak IV: trigonelline-like compound fraction contained a trace amount of N¹-methylnicotinamide. Fractions 20 to 30 were pooled and evaporated to dryness. The procedure was repeated 20 times. This residue was dissolved with 25 ml of water.

Dowex 50W-X12 and SP-Sephadex C-25 column chromatographic elution pattern. The solution obtained in B (see Fig. 1B) was contaminated with a small amount of N¹-methylnicotinamide and N¹-methyl-4-pyridone-3-carboxamide. Furthermore, a 3 to 4 ml portion of solution (Peak IV) was applied to a SP-Sephadex C-25 column (25 × 900 mm) and 10 ml fractions were collected. The solutions of fraction numbers 53 to 70 were pooled and evaporated to dryness. This treatment was repeated 8 times. Finally, the isolated compound was crystallized from ethyl alcohol, white rod-like crystals being obtained. Figure 2 shows a comparison of the ultraviolet spectra of the authentic compound and the compound isolated from urine. The
Fig. 2. Ultraviolet spectra of authentic trigonelline and the material isolated from rat urine as measured on a Shimadzu UV-300 double-beam spectrophotometer. Compound dissolved in H$_2$O (---); in 1N HCl (----); in 1N NaOH (-----). The concentration of authentic compound was $5 \times 10^{-5}$ M.

Fig. 3. Infrared spectra of authentic trigonelline (A) and the compound isolated from rat urine (B). Spectra were determined with a Shimadzu IR-300 grating infrared spectrophotometer from a KBr pellet prepared with a microsampling kit.

absorption spectra of authentic Tg in H$_2$O exhibited a maximum at 265 nm and a shoulder in the neighborhood of 268 to 270 nm; in 1N HCl, a 265 nm maximum and a 268 to 270 nm shoulder; and in 1N NaOH, the maximum at 265 nm and the 268 to 270 nm shoulder disappeared. The spectra of the isolated compound in H$_2$O,

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Fig. 4. Mass spectra of authentic trigonelline and the compound isolated from rat urine. Mass spectra were determined with a Shimadzu GL-MS 7000 gas chromatography-mass spectrometer with the use of the direct inlet probe. The ion source temperature was 290°C, ionization current was 60 μA, probe temperature was 111°C, electron energy was 70 eV, and acceleration voltage was 3.0 V.

HCl and NaOH are almost identical with those of the authentic compound.

Further evidence of the identity of the isolated compound is provided by comparison of infrared spectra as shown in Fig. 3. The infrared absorption spectrum of authentic Tg in Fig. 3A showed a medium absorption band at 1377 cm\(^{-1}\) and a strong absorption band at 1665 cm\(^{-1}\). These two bands, at 1377 cm\(^{-1}\) and 1665 cm\(^{-1}\), are characteristic of the N-CH\(_3\) functional group and the carbonyl or ketone functional group or both, respectively. The infrared spectrum in Fig. 3B shows that the isolated compound has similar spectrum corresponding to the same functional group. The infrared spectrum of the isolated compound is almost identical with that of the authentic compound.

The gas-mass spectra in Fig. 4 are also nearly identical with those of authentic Tg and the compound isolated from urine. The cleavage of both authentic and isolated Tg formed the same fragments at \(m/e\) 173, 93 and 77.

Elemental analysis:

|            | C      | H     | O       | N       |
|------------|--------|-------|---------|---------|
| Authentic TG | 61.34  | 5.69  | 23.04   | 10.10   |
| Isolated compound | 61.22  | 5.33  | 22.96   | 10.25   |

The elemental analytical values of both samples are almost identical.

The \(R_f\) value of the isolated compound corresponded to that of authentic Tg.

From these results it was clearly demonstrated that the isolated compound
from rat urine was identical with the authentic Tg.

**DISCUSSION**

In the present investigations it was shown that Tg is a urinary metabolite of nicotinic acid in rat. Evidence was thus provided that the single- and double-labeled Tg were isolated from rats with $^{14}$C-NiA and/or $^3$H-SAM *in vivo* injected into the portal vein and they were identified by chromatographic behavior, ultraviolet absorption pattern and cocrystallization with the authentic carrier. Further evidence was found that Tg was isolated in normal rat urine and crystallized from ethyl alcohol, and was identified by ultraviolet spectra, infrared spectra, gas-mass spectrometry, elemental analysis, chromatographic behavior and melting point. These results conflict with other findings that nicotinic acid itself is not converted into Tg in rats and mice (13–16), although Ackermann (2) reported the isolation of Tg from the urine of dog given high doses of nicotinic acid (9 g in 5 days). Mason and Kodicek observed the excretion of Tg in rats fed on basal diet, however, they stated that a source of urinary Tg was dietary niacytin (17). Tarr showed that Tg is excreted in normal human urine (18). In studies with labeled nicotinic acid, labeled Tg was not found in urine, although it was possibly not investigated as such (e.g. Bonavita et al. (9), Chaykin et al. (21), Greengard et al. (22), Petrack et al. (23) and Lee et al. (10)). This failure to identify this Tg metabolite may be due to the fact that it is extracted in relatively small amounts and that in butanol-water (9) and butanol-ammonia solvent systems (21–23) most commonly used for paper chromatography of nicotinic acid metabolites, Tg has approximately the same $R_f$ as N$^1$-methylnicotinamide.

On the other hand, the enzymatic synthesis of Tg from nicotinic acid is not known in rats and other mammals, although it was demonstrated that TG containing $^{14}$C and $^3$H was identified in urine when $^{14}$C-NiA and $^3$H-SAM were injected into the portal vein of rats. In order to support the above evidence, we investigated with radioassay whether Tg was synthesized by rat liver enzyme. As a result, the labeled Tg is found to be synthesized in the same manner to form N$^1$-methylnicotinamide (20).

The results presented clearly indicate that Tg is a metabolite of nicotinic acid in normal rat. However, the physiological significance of Tg remains obscure.

The establishment of a simple method for the determination of daily excreted Tg in rat urine will be reported later.

The authors wish to thank the Analytical Center of Shimadzu Seisakusho Ltd., for measuring the Gas-Mass for study.

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