Identification of Pyridoxine 3-Sulfate, Pyridoxal 3-Sulfate, and N'-Methylpyridoxine as Major Urinary Metabolites of Vitamin B₆ in Domestic Cats*

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Preliminary studies of vitamin B₆ metabolism in three adult domestic cats detected very little pyridoxic acid in the urine. At oral doses of 49 to 490 μmol of [¹⁴C]pyridoxine hydrochloride, 50% of the excreted dose occurred as pyridoxine 3-sulfate and 25% as N'-methylpyridoxine. The identity of these two metabolites was confirmed by isolation from urine and comparison with known compounds. A third compound was identified as pyridoxal 3-sulfate on the basis of chromatographic behavior and fluorescent properties before and after hydrolysis. At pyridoxine intakes of 0.97 μmol/day, the concentration of pyridoxal 3-sulfate in the urine sometimes exceeded the concentration of pyridoxine 3-sulfate. Pyridoxic acid remained a minor urinary metabolite at pyridoxine intakes ranging from 0.97 to 490 μmol/day. Although sulfation of phenol groups and methylation of the ring nitrogen are well-known detoxication reactions, this appears to be the first time such reactions have been observed in normal metabolism of vitamin B₆. These observations provide further evidence of the diversity of vitamin B₆ metabolism between species. While such diversity complicates the extrapolation of data from animal studies to humans, it does provide a variety of models for examining the influences of various factors on vitamin B₆ metabolism.

In a study of the plasma vitamin B₆ content of various animals, Coburn et al. (1) found that the pyridoxal phosphate concentrations in cats averaged 2400 nM, which was over five times higher than in the other species studied. Whyte et al. (2) reported that plasma pyridoxal phosphate in subjects with hypophosphatasia averaged 1174 nM. Analysis of the plasma remaining from the animal studies revealed that, like the hypophosphatasia patients, the cats had low alkaline phosphatase activity. Since the cats do not exhibit any bone disease, we initiated a study of the kinetics of vitamin B₆ metabolism in cats. When preliminary urine collections revealed very little pyridoxic acid, the following studies of urinary metabolites were conducted.

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MATERIALS AND METHODS
The current data was obtained from adult, male, mixed breed cats weighing about 4 kg. They were fed either commercial cat food or a vitamin B₆-deficient purified diet (Table 1). [¹⁴C]Pyridoxine was donated by Hoffmann-La Roche and purified by cation-exchange liquid chromatography (3). Oral supplements of 0.97 to 490 μmol of pyridoxine hydrochloride/day were provided on small pieces of tuna.

To isolate the major metabolite (compound X) the urine was adjusted to pH 2.5, centrifuged, and filtered. Up to 50 ml was placed on a cation-exchange column (11 × 2 cm, Dowex 50W-X8, 100–200 mesh NH₄⁺ form), followed by 50 ml of water. The total effluent, which contained compound X, was adjusted to pH 8.4, filtered, and placed on an anion-exchange column (32 × 1 cm, Dowex 2-X8, 50–100 mesh, OH⁻ form). The column was washed with 50 ml of water and 86 ml of 0.1 M acetic acid. Compound X was then eluted with 0.1 N hydrochloric acid. Its location was monitored by absorbance at 277 nm at pH 1. The effluent was adjusted to pH 7 and concentrated to 12 ml under vacuum at 40 °C, adjusted to pH 3.7, and applied to a Florisil column (28 × 1 cm, 60–100 mesh, Fisher) which had been equilibrated in 0.1 N hydrochloric acid and washed with water. Compound X eluted in the early effluent. The fraction containing compound X was adjusted to pH 7 with sodium hydride, evaporated to dryness, triturated with benzene, and extracted into ethanol. The solution was adjusted to a reading of 4 on a pH meter by adding 7.5 N hydrogen chloride in ethanol. After centrifugation the supernatant solution was removed and adjusted to a reading of 10.5 on a pH meter with a saturated solution of barium hydroxide in ethanol. The solution was evaporated to dryness, redissolved in 2.5 ml of water and applied to a Sephadex G-15 column (Pharmacia P-L, Biochemicals, 25 × 2 cm). The metabolite eluted in the 23-35-ml fractions. The fractions were combined and evaporated to dryness. Metabolite X was redisolved in ethanol and crystallized by adding ethyl ether. The solid was washed with ether and vacuum-dried under vacuum.

Metabolite Y was retained on the Dowex 50 column. The column was washed sequentially with 50 ml of water, 100 ml of 0.1 M potassium acetate, pH 6.0, and 50 ml of water. Metabolite Y was eluted in 100 ml of 3 N NH₄OH. The eluate was concentrated to 20 ml, adjusted to pH 9.2, and applied to an anion-exchange column (11 × 2 cm, Dowex 2-X8, 200–400 mesh, OH⁻ form), followed by 25 ml of water. The total effluent was concentrated to 17 ml, adjusted to pH 5, and applied to a Florisil column (3 × 1 cm) which had been equilibrated in 3 N ammonium hydroxide and washed with water.

The location of compound Y was monitored by measuring absorbance at 330 nm at pH 7. This effluent was evaporated to dryness, triturated with benzene, and redissolved in ethanol. The solution was adjusted to a reading of 1 on a pH meter by adding 100 μl of 4.5 N hydrochloric acid. The sample was evaporated to dryness, redissolved in hot ethanol, and crystallized by concentrating and cooling the solution. The white solid was washed with ether and vacuum-dried.

Pyridoxine 4′-diacetate hydrochloride (4) — Pyridoxine hydrochloride (13.0 g, 0.0632 mol) was dissolved in 25 ml of glacial acetic acid. Acetyl chloride (11 ml, 0.185 mol) was added, and the mixture was heated at 48–50 °C for 2 h under reflux. Then the reflux condenser was removed and heating was continued for 30 min. After cooling, the product was crystallized by addition of 20 ml of ethyl acetate and 5 ml of heptane. The product was collected by filtration, washed with heptane, and dried, yielding 16.8 g (92%).
Pyridoxine 3-Sulfate (I)—Pyridoxine 4,5-diacetate hydrochloride (2.9 g, 11.5 mmol) plus 1.4 ml of triethylamine (10 mmol) were dissolved in 25 ml of dry chloroform. Following addition of 1.6 g of sulfur trioxide-trimethylamine complex (11.5 mmol, Aldrich) and 1.4 ml triethylamine, the mixture was stirred overnight at room temperature. The progress of the reaction was monitored by following the loss of absorbance at 323 nm at pH 7. Upon completion, the mixture was extracted with two 40-ml portions of water. After addition of 3 ml of 18 M sodium hydroxide to the combined aqueous extracts, the mixture was heated in a boiling water bath for 15 min to hydrolyze the acetate esters. The solution was evaporated to dryness and triturated with benzene. The residue was extracted with ethanol and the extract was concentrated almost to dryness. The concentrate was brought to 20 ml with water, adjusted to pH 5, and placed on a cation-exchange column (13 × 2 cm, Dowex 50W-X8, 100–200 mesh, H+ form) followed by 20 ml of water. The total effluent was adjusted to pH 7, evaporated to dryness, and triturated with benzene. The residue was dissolved in ethanol and recrystallized by addition of 2-propanol (ZI) to yield 270 mg of the barium salt. N-Methylpyridoxine iodide (II) was synthesized by reacting pyridoxine with iodomethane (5).

Ultraviolet and infrared spectra were determined on Shimadzu model 160 and Beckman Acculab spectrophotometers, respectively.

**TABLE I**

| Vitamin-free casein | 31.6 |
| Melted turkey fat (0.02% butylated hydroxytoluene) | 24.7 |
| Corn starch | 19.8 |
| Sucrose | 14.8 |
| Salt mix<sup>e</sup> | 3.9 |
| Vitamin mix<sup>f</sup> | 2.2 |
| Sodium acetate | 1.7 |
| Di-Methionine | 1.0 |
| Taurine | 0.1 |

<sup>e</sup> See Morris (1976) in Ref. 14.

<sup>f</sup> Vitamin Diet Fortification Mixture without vitamin B<sub>6</sub> (Catalog No. 904655, Nutritional Biochemicals, Cleveland, OH).

RESULTS AND DISCUSSION

Following intakes of [1<sup>4</sup>C]pyridoxine hydrochloride ranging from 0.97 to 490 µmol, only 2–3% of the activity appeared in the urine as pyridoxic acid even though intakes of 49 to 490 µmol about 70% of the dose was excreted within 24 h. Cation-exchange liquid chromatography revealed that about 50% of the excreted dose eluted faster than the normal vitamin B<sub>6</sub> metabolites, suggesting that it (compound X) was more negatively charged than the pyridoxal 5'-phosphate at pH 1.7. About 20–25% of the excreted dose eluted after the normal vitamin B<sub>6</sub> metabolites, indicating that this compound (Y) might have a tightly bound positive charge. Compound X was stable in base but in acid was hydrolyzed to pyridoxine and pyridoxal. The relative amounts of pyridoxine and pyridoxal depended on the pyridoxine intake. At doses of 0.97 µmol/day the amount of the pyridoxal compound sometimes exceeded the amount of the pyridoxine form but the quantity was too small to isolate. At higher intakes, pyridoxine sulfate became the major product with little increase in the amount of pyridoxal sulfate. The chromatographic and chemical properties of compound X were similar to those we had previously encountered with the 3-sulfate esters of 4'- and 5'-deoxypyridoxines (6, 7). After isolation and fusion of compound X with sodium, a positive nitroprusside test (8) for sulfur was obtained. Compound X ran with pyridoxine-3-sulfate in liquid chromatography and in five solvent systems (Table II) in thin layer chromatography on silica gel. Neither compound X nor pyridoxine-3-sulfate reacted with the p-nitroaniline spray for phenols (9) until hydrolyzed.

The sulfate esters are difficult to isolate in pure form because their sodium salts are very hygroscopic. The zwit- tieron form is less hygroscopic but is readily hydrolyzed during drying if any excess acid is present. In the current study we attempted to avoid these problems by isolating both the synthetic and biological compounds as barium salts. The barium salt of the synthetic compound crystallized nicely; however, treatment of the urine extract with barium hydroxide gave an impure product. Final purification using G-15
Sephadex unexpectedly resulted in separation of the barium ion and regeneration of the zwitterion form. This could be dried since no acid was present. The salt and zwitterion forms have distinct infrared spectra. Passage of the synthetic barium salt through the Sephadex column yielded infrared and ultraviolet spectra identical to that of compound X (Fig. 1 and Table IV).

Compound Y was not hydrolyzed by acid or base. Its behavior in liquid and thin layer chromatography (Table III) was identical to that of N-methylpyridoxine. Neither Y nor N-methylpyridoxine gave a positive test with p-nitroaniline. Their ultraviolet (Table IV) and infrared (Fig. 2) spectra were similar. Pyridoxine added to cat urine and allowed to stand 2 weeks at 4°C was almost completely recovered unchanged, indicating that these metabolites were produced prior to excretion.

We had previously identified 3-sulfate esters as metabolites of 4'-deoxypyridoxine in the urine of several species, including cats (10); however, this appears to be the first case where sulfate esters have been identified as the major urinary metabolites of pyridoxine. It also appears to be the first time that N-methylpyridoxine has been identified as the metabolite of vitamin B6 metabolism. A single urine specimen collected from a bobcat suggested that these metabolites may occur in other types of cats.

Cats have low concentrations of pyridoxic acid in both plasma and urine. This may reflect limited capacity to oxidize pyridoxal, thus forcing the utilization of alternate pathways. Another possibility is that the sulfation and methylation are so rapid that little excess is available for oxidation. Further work will be necessary to distinguish between these choices. This is another example of marked metabolic differences between the cat and other monogastric mammalian species. Altered tryptophan metabolism is demonstrated by the observation that, whereas rats, dogs, and swine excreted 12 to 40% of an oral tryptophan dose as identified urinary metabolites, cats excreted only 0.07 to 1.3% (11). Cats are also unable to synthesize adequate taurine (12).

Sulfate esters have been identified as a metabolite of 5'-deoxypyridoxine in humans (10). At least nine unidentified minor metabolites of vitamin B6 were detected in human urine after oral administration of [14C]pyridoxine (13). It is possible that these may have included sulfated and methylated derivatives. It is clear that much remains to be learned about vitamin B6 metabolism in vivo.

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REFERENCES