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IDENTIFICATION OF 5'-DEOXYPYRIDOXINE-3-SULFATE AS THE MAJOR URINARY METABOLITE OF 5'-DEOXYPYRIDOXINE IN RATS WITH COMMENTS ON THE INHIBITION OF ARYLSULFATASE ACTIVITY AND MANGANESE DIOXIDE OXIDATION BY NEIGHBORING GROUPS

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SUMMARY: The major urinary metabolite of 5'-deoxypyridoxine in rats was shown to be identical to 5'-deoxypyridoxine-3-sulfate but different from 5'-deoxypyridoxine -4'-sulfate in its ultraviolet and infrared spectra, its migration in thin-layer chromatography, and its behavior in acid and base. Previous identification of the metabolite as 5'-deoxypyridoxine-4'-sulfate by other workers was based on its failure to be hydrolyzed by arylsulfatase and to be oxidized by manganese dioxide. We have now demonstrated that ortho-methyl groups inhibit arylsulfatase and that ortho-sulfate groups inhibit oxidation by manganese dioxide. Therefore, we conclude that under our conditions the major metabolite of 5'-deoxypyridoxine in the rat was the 3-sulfate.

Shane and Snell (1) found that injected 5'-deoxypyridoxine was rapidly distributed in rat tissues, then almost quantitatively excreted, apparently because it cannot be phosphorylated. Properties of the major urinary metabolite of this component suggested that it was 5'-deoxypyridoxine-4'-sulfate. Location of the sulfate on the primary hydroxyl at the 4'-position rather than on the phenolic hydroxyl at the 3-position was surprising since conjugation of phenols with sulfate is commonly observed (2) and since we had identified the 3-sulfate as the major urinary metabolite of 4'-deoxypyridoxine (3). Therefore, we reexamined the metabolism of 5'-deoxypyridoxine and in the

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course of that investigation discovered some major effects of neighboring groups on both arylsulfatase activity and manganese dioxide oxidation reactions.

METHODS

5'-deoxypyridoxine was synthesized according to Korytnyk and Ikawa (4) except that the reaction with thionyl chloride was refluxed for 2 hours. 5'-deoxypyridoxine-4'-acetate was prepared by reacting 950 mg 5'-deoxypyridoxine hydrochloride in 2 ml glacial acetic acid and 0.4 ml acetyl chloride (5). 5'-deoxypyridoxine-3-sulfate was prepared as described for 4'-deoxypyridoxine-3-sulfate (3) by reacting the 4'-acetate derivative with chlorosulfonic acid in pyridine and removing the acetate by base hydrolysis. Sulfate esters of 3-hydroxy-pyridine and 6-methyl-3-hydroxy-pyridine (Aldrich Chemical Co., Milwaukee, WI) were prepared using chlorosulfonic acid in a similar manner.

Since the migration of acyl groups from the 3 to the 4'-position (6) made it difficult to protect the phenol group selectively during synthesis of the 4'-sulfate, we took advantage of our observation that in reaction with chlorosulfonic acid the 4'-position is favored over the 3-position. Chlorosulfonic acid (0.35 ml) was added dropwise to 15 ml pyridine containing 1 g 5-deoxypyridoxine hydrochloride. During the addition the mixture was stirred and maintained at 0° C. After 45 minutes another 0.35 ml chlorosulfonic acid was slowly added at 0° and stirring continued at room temperature overnight. The progress of the reaction was monitored by thin-layer chromatography and by measuring the absorbance at 320 nm at pH 7, which is characteristic of the phenol group. The timing and amount of chlorosulfonic acid were adjusted to minimize both the amount of unchanged starting material in the reaction mixture and the loss of absorbance at 320 nm at pH 7. The reaction mixture was concentrated in vacuo at 35° C and the product purified by crystallization from ethanol and ether.

Assignment of the sulfate to the 4'-position was based on a positive test with diazotized p-nitroaniline (7) and retention of the absorption maximum at 320 nm at pH 7 both of which indicate that the phenol group remained free. Further evidence of substitution at the 4'-position was the spontaneous formation of a water insoluble polymer when we attempted to produce the sodium salt. Similar polymerizations have been reported for other 4'-derivatives (8, 9).

Phenyl sulfates of o-cresol; m-cresol; p-cresol; 2,4-dimethylphenol; 2,5-dimethylphenol; 2,6-dimethylphenol; 3,4-dimethylphenol; 3,5-dimethylphenol, 2,6-dimethyl 4-nitrophenol (Aldrich); and 2,3-dimethylphenol (City Chemical Corp., New York, N.Y.) were prepared by stirring at room temperature in chloroform with a slight excess of sulfur trioxide trimethylamine complex (Aldrich) in the presence of triethylamine. The progress of the reaction was followed spectrophotometrically by measuring the decrease in absorbance as the phenol group was esterified (Table I). The reactions required up to a week to reach completion. The crude product was precipitated by neutralizing with 50 percent aqueous potassium hydroxide. The material was purified by recrystallization from water or water/methanol mixtures until the potassium analysis agreed with the calculated value and there was no significant absorbance at the appropriate maximum (Table I). Most of the sulfates could be dried in air at 110° C. However, the o-methyl and 3,5-dimethyl derivatives appeared to contain one mole of water of crystallization which could not be readily removed without causing hydrolysis of the product.

Table 1: Molar extinction coefficients for various phenolic compounds in 0.1 N potassium hydroxide.

	nm	E
Phenol	285	2714
Methylphenols		
o-	290	3150
m-	290	2515
p-	295	2444
Dimethylphenols		
2,3-	290	2992
2,4-	295	3028
2,5-	290	3560
2,6-	285	3743
3,4	295	2853
3,5	290	2681

Manganese dioxide was prepared and used under the conditions specified by Shane and Snell (personal communication). Oxidation with pyridinium chlorochromate (Aldrich) was also tested under the conditions specified by the manufacturer. Aryl sulfatases (E.C.3.1.6.1) from limpet, Helix pomatia, abalone entrails, and Aerobacter aerogenes were obtained from Sigma Chemical Co. (St. Louis, MO) and assayed using the conditions specified by the manufacturer.

Two adult male rats from the Wistar colony at the Biochemistry Department, Purdue University (West Lafayette, IN) were fed the synthesized 5'-deoxypyridoxine·HCl in the drinking water at a concentration of 2 mg/ml in conjunction with a B_6 deficient diet. Urine was collected for 5 days and processed using ion-exchange chromatography as described previously for studies with 4'-deoxypyridoxine (3).

RESULTS AND DISCUSSION

As reported by Shane and Snell, 5'-deoxypyridoxine is rapidly excreted. Approximately 80% of the excreted material showed no absorbance at 320 nm at pH 7 before acid hydrolysis and gave no positive test for a phenol group with diazotized p-nitro-aniline (7). The material was stable to base, but following acid hydrolysis it moved with 5'-deoxypyridoxine on thin-layer chromatography and showed a positive test for a free phenol group. These results were consistent with the properties we observed previously for 4'-deoxypyridoxine-3-sulfate. Synthesized 5'-deoxypyridoxine-3-sulfate showed an identical infrared spectrum (Figure 1) and ran with the metabolite in four solvents on silica gel (Table 2).

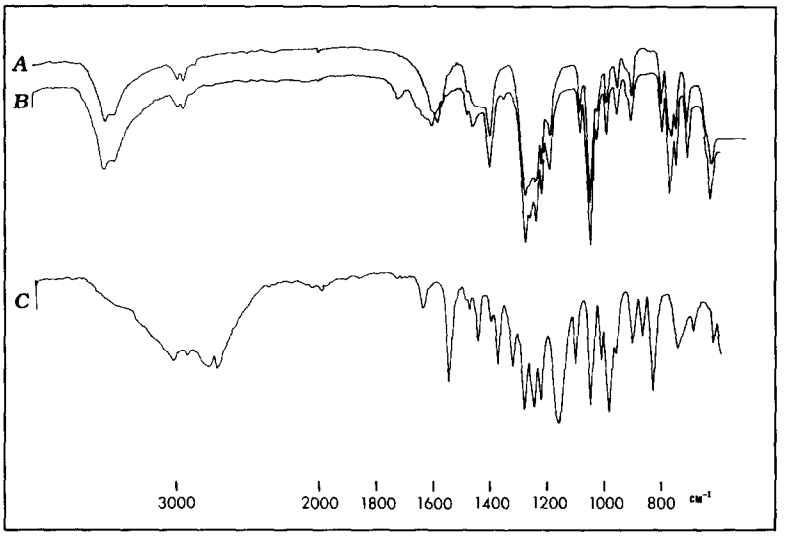

Figure 1: Infrared spectra.

A. 5'-deoxypyridoxine-3-sulfate (sodium salt).
B. Rat metabolite (sodium salt).
C. 5'-deoxypyridoxine-4'-sulfate.

Table 2: R_f values of 5'-deoxypyridoxine (DOP) compounds on silica gel plates.

Solvent*	5-DOP	5-DOP-4'-SO_4	5-DOP-3-SO_4
A	.16	.90	.95
B	.85	.95	.38
C	.58	.64	.69
D	.60	.40	.50

*A-water
B-Chloroform:methanol (75:25)
C-95% ethanol
D-2-butanol:water:concentrated ammonium hydroxide (55:18:2)

It should be noted that the spectra contained in Figure 1 were obtained from the sodium salt of the 3-sulfate and from the zwitterion of the 4'-sulfate. This was necessitated by the fact that phenyl sulfates are stable in base (10) but readily hydrolyzed in acid (11), while the 4'-sulfate, like some other 4'-derivatives (8, 9), is more stable in acid but spontaneously polymerizes in base. Therefore, it would have been very difficult to obtain pure

Table 3: Relative activity of aryl sulfatases toward pyridyl-3-sulfate derivatives taking p-nitrocatechol sulfate as 100%.

Substrates	Enzyme	
	Limpet	Helix
p-nitrocatechol sulfate	100.0	100.0
p-nitrophenyl sulfate	18.9	100.0
3-pyridyl sulfate	35.4	51.9
6-methyl-3-pyridyl sulfate	5.2	41.2
4'-deoxypyridoxine-3-sulfate	<1.0	<1.0
5'-deoxypyridoxine-3-sulfate	<1.0	<1.0

samples of the 3- and 4'-sulfate in the same ionic form. In view of this marked difference in chemistry, the fact that the behavior of the metabolite in acid and base resembled that of a phenyl sulfate is strong evidence for location of the sulfate in the 3-position. Shane and Snell (1) noted that the half-time for hydrolysis of their compound in 0.1 M HCl at 100° was 4.8 min compared with 28 min for pyridoxal -5'-sulfate. This ready cleavage by acid is consistent with a phenyl sulfate structure.

Shane and Snell (1) cited the inability of arylsulfatase to hydrolyze their metabolite as evidence that their metabolite was not a phenyl sulfate. However, we found that the 3-sulfates of both 4'- and 5'-deoxypyridoxine failed as substrates for arylsulfatase (Table 3). These compounds differ from the commonly used substrates in that they contain a pyridine ring rather than a benzene ring and are highly substituted. Since the ability of arylsulfatase to hydrolyze simpler pyridyl sulfates (Table 3) suggested that the presence of the pyridine ring alone was not sufficient to prevent activity, we investigated the possible role of substitution. It is clear that for each of the sulfatases tested dimethyl substitution ortho to the phenol group essentially eliminated arylsulfatase activity even in a good substrate such as p-nitrophenyl sulfate (Table 4). The Limpet enyzme was not included in Table 4 because it showed no activity against any of the substrates listed in Table 4 other than p-nitrocatechol sulfate and p-nitrophenyl sulfate. Since none of the enyzmes tested were able to hydrolyze pyridyl or phenyl sulfates sur-

Table 4: Relative activity of arylsulfatases toward substituted phenylsulfates using phenylsulfate as 100%.

Subtrates	Enzyme		
	Helix	Abalone	Aerobacter
4-nitrocatechol sulfate	2750	3840	230
4-nitrophenyl sulfate	2580	4060	278
2,6-dimethyl-4-nitrophenyl sulfate	<5	< 30	< 1
phenyl sulfate	100	100	100
Methylphenyl sulfates			
o-	14	130	24
m-	638	347	161
p-	412	237	121
Dimethylphenyl sulfates			
2,3-	20	176	25
2,4-	38	209	16
2,5-	14	149	3
2,6-	< 1	< 10	< 1
3,4-	717	397	103
3,5-	1170	540	18

rounded by methyl groups, arysulfatase cannot be used to distinguish between 5'-deoxypyridoxine-3-sulfate and 5'-deoxypyridoxine-4'-sulfate.

The second major piece of evidence which Shane and Snell presented for the location of the sulfate on the 4'-position was the failure of their metabolite to undergo oxidation with manganese dioxide suggesting that the 4'-position was blocked. Using their oxidation procedure, we found that although pyridoxine and 5'-deoxypyridoxine were easily oxidized as evidenced by a change in the spectrophotometric readings and a positive phenylhydrazine test for aldehydes, the rat metabolite, 5'-deoxypyridoxine-4'-sulfate, and 5'-deoxypyridoxine-3-sulfate all failed to show evidence of oxidation. Pyridoxine-5'-phosphate was oxidized but much more slowly than pyridoxine. Tests at room temperature with pyridinium chlorochromate, another mild oxidizing agent, confirmed that 5'-deoxypyridoxine-3-sulfate was much more resistant to oxidation than 5'-deoxypyridoxine. Therefore, the manganese dioxide oxidation test is not a satisfactory method of distinguishing between the 3- and 4'-sulfate derivatives.

Based on these results, we conclude that the major urinary metabolite of both 4' and 5'-deoxypyridoxines in the rat is the 3-sulfate. Since the

chemical properties of the 3-sulfate coincide with those used by Shane and Snell (1) to infer conjugation at the 4'-position, there is no longer any need to postulate formation of the 4'-sulfate as a major excretory product.

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