SYNTHESIS OF DEUTERATED VITAMIN B_6 COMPOUNDS

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SUMMARY

3-Hydroxy-4-(hydroxymethyl)-5-(hydroxymethyl-d_2)-2-methylpyridine (pyridoxine-d_2) was prepared by reduction of α^4-3-O-isopropylidene-5-pyridoxic acid with lithium aluminum deuteride. Deuterium was inserted in the 2-methyl group using base catalyzed exchange between deuterium oxide and N-benzyl pyridoxine. Pyridoxine-d_2 was converted to pyridoxal, pyridoxamine, pyridoxic acid, pyridoxine phosphate, pyridoxal phosphate, and pyridoxamine phosphate. After acetylation the nonphosphorylated forms could be analyzed by gas chromatography-chemical ionization mass spectroscopy.

Key words: Vitamin B_6, Deuterium.

INTRODUCTION

The complex chemistry and multiple forms of vitamin B_6 present serious analytical problems which in turn complicate in vivo studies of vitamin B_6 metabolism. The compounds described here were produced in connection with an attempt to use stable isotope tracers to improve the assessment of vitamin B_6 status in man. Syntheses of pyridoxine labeled with ^{14}C at the hydroxymethyl groups or the 4 and 5 ring positions have been achieved by Diels Alder condensations between appropriately labeled diethyl fumarate and 4-methyl-5-ethoxyoxazole (1, 2). The 5-hydroxymethyl group has been labeled specifically (3) and routes for ^{14}C labeling of other positions have been suggested (4). Insertions of ^{15}N in the 4-aminomethyl group (5) of pyridoxamine and ^{32}P at the 5'-position of pyridoxal phosphate (6, 7) have also been reported. Pyridoxine labeled randomly with tritium by the Wilzbach gas exposure technique (8) is commercially available and has been converted to pyridoxal-5'-phosphate by incubation with E. coli (9). More specific tritium labeling has been achieved by reducing 4-pyridoxic acid lactone, 5-pyridoxic acid

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lactone or diethyl 5-hydroxy-6-methylcinchomeronate with lithium aluminum tritide (2, 10) or by reducing pyridoxal-5'-phosphate with sodium borotritide (6). Labeling with deuterium was noted in the 2-methyl group of N-methylpyridoxine during proton magnetic resonance studies (11) and in the 2-methyl, 4-hydroxymethyl and 6-hydrogen of 4'-deoxypyridoxine following treatment with deuterated hydrazine (12). We now report specific insertion of deuterium into the 5-hydroxymethyl group using lithium aluminum deuteride reduction of isopropylidene-5-pyridoxic acid (Figure 1). Deuterium was also inserted into the 2-methyl group by base catalyzed exchange of N-benzylpyridoxine in deuterium oxide (Figure 1). Other derivatives were then prepared from the deuterated pyridoxine (Figure 2).

RESULTS AND DISCUSSION

Vitamin B_6 compounds present several problems to the synthetic chemist. Their highly polar amphoteric character limits their solubility in most solvents other than water. Another challenge is the lability of the aldehyde group as illustrated by the 58% yield for preparation of the oxime (9) from pyridoxine without isolating the intermediate aldehyde form compared with 26% yield for the isolation of the aldehyde itself. Although the procedures presented here have not all been refined to give maximum yields, the routes selected are sufficiently inexpensive that the low yields of some steps are not a major obstacle. For example, the cost of labeled reagents was approximately \$2.50 per gram of pyridoxine-d_2 hydrochloride and about \$60 per gram of pyridoxine-d_5 hydrochloride.

As noted by Korytnyk (13) a high concentration (about 15%) of hydrogen chloride is essential to achieve a high yield of the α^4-3-O-isopropylidene derivative (1). At lower concentrations the α^4, α^5 compound may be obtained. Since the solubility of hydrogen chloride is very temperature dependent, the acetone must be thoroughly chilled. The solution will turn dark orange

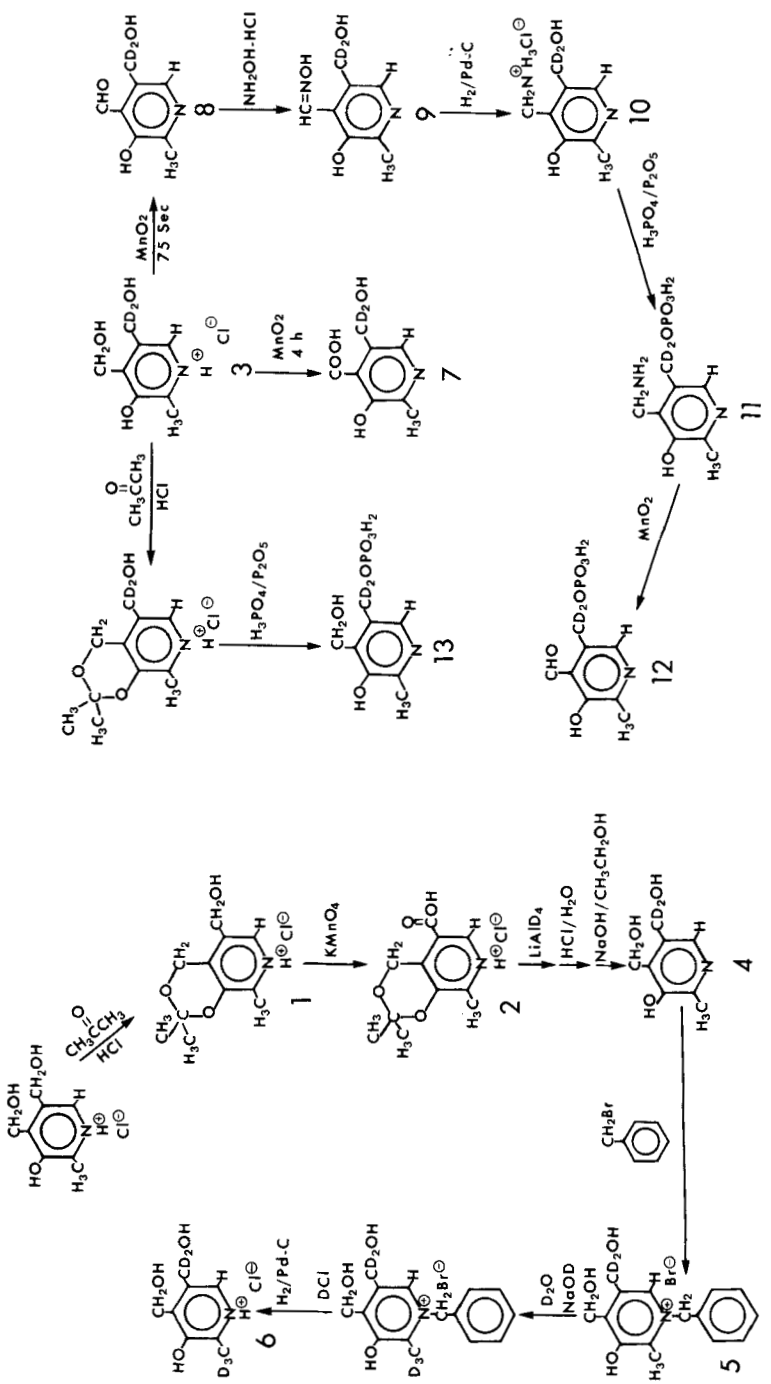

Fig. 1. Synthesis of deuterated pyridoxine.

Fig. 2. Synthesis of labelled B_6 derivatives.

after a few hours in the cold. We have monitored the reaction and when necessary have added more hydrogen chloride to force the reaction to completion. Under these conditions we have routinely obtained yields approaching 100%.

Frequently, esterification of carboxyl groups is necessary to obtain satisfactory yields from lithium aluminum hydride reductions. In the case of isopropylidene 5-pyridoxic acid esterification yielded little improvement and therefore was omitted.

Korytnyk and Singh (11) had noted rapid hydrogen exchange at the 2'-position of N-methylpyridoxine in 1 N sodium hydroxide. We took advantage of this observation to label the 2'-position using the N-benzyl derivative since the benzyl group can be removed more readily than the methyl group. Because of the speed of the exchange reaction, it is important to avoid contact with H_2O until after the base has been neutralized. Our use of deuterium chloride after the exchange reaction resulted in a mixture of the chloride and bromide salts and necessitated additional purification steps. It would be preferable in the future to use deuterium bromide thus producing pure pyridoxine hydrobromide.

Some related N-benzyl derivatives had previously been produced as intermediates in the total synthesis of pyridoxine from ethyl-N-benzyl-N-1-carbethoxyethylaminomethylene-succinate (14). However, the current preparation appears to be the first report of direct N-benzylation of pyridoxine. Before adopting the exchange approach for labeling the 2'-position, we attempted to use the electrochemical synthesis of Elming and Clauson-Kaas (15). These authors reported efficient incorporation of acetate into the 2-position. However, we were unable to duplicate the high yields they obtained.

In the conversion of pyridoxine to pyridoxal, it is important to control the oxidation step as closely as possible to prevent excessive conversion to

4-pyridoxic acid lactone. With as little as 75 second shaking there was a substantial amount of lactone which must be eliminated by repeated washings and crystallizations.

Lloyd's reagent is a standardized magnesium silicate preparation which until recently was used commonly as an adsorbent in clinical laboratories. As noted in the experimental section, we found it very useful in purifying the products from these syntheses. Unfortunately, to our knowledge Lloyd's reagent is no longer manufactured in the United States. We have not found the fullers earths now offered as substitutes to be as reliable as the original reagent.

In an attempt to improve the synthesis of the phosphate esters we tried several procedures. Nakagawa et al. (16) reported high yields using intermediates obtained by condensation between pyridoxal and a secondary amine. However, the necessity of making the condensation product coupled with problems in purifying the phosphorylated compound offset the high yield of the phosphorylation step. Moffatt and Khorana (17) found tetra(p-nitrophenyl) pyrophosphate to be a useful phosphorylating agent for a variety of compounds although they did not report testing any vitamin B_6 compounds. We were unable to obtain any reaction with vitamin B_6 derivatives. Therefore, we adopted the phosphoric acid-phosphorus pentoxide mixture used by Peterson and Sober (18). Isolation of the product from the excess phosphate is one of the major problems with this approach. We did not become aware of the work of Stock, et al. (6) until later. Their use of a stoichiometric amount of phosphorus oxychloride might simplify the subsequent purification of the product.

While the isotopic compositions reported here are acceptable, more recent results suggest that there was a slight memory effect with the mass spectrometry procedures used in these measurements. As a result, the iso-

topic purity is probably better than has been specified here. For example, preliminary tests with modified procedures indicate that pyridoxine-d_2 is about 95% d_2 rather than 85%. Therefore, the isotopic composition values reported in the experimental section are minimal values.

The availability of these compounds will permit use of isotope dilution techniques to verify current analytical procedures for vitamin B_6 and should facilitate more detailed studies of vitamin B_6 metabolism in vivo in man.

EXPERIMENTAL

Elemental analyses were within 0.6% of the calculated values. Melting points are uncorrected. All labeled B_6 vitamers ran with authentic unlabelled samples on both thin layer and high performance liquid chromatography. Isotopic abundances were determined by gas chromatography-isotope ratio-mass spectrometry on the acetate derivatives using NH_3 chemical ionization. A detailed description of these techniques will be published elsewhere.

α^4,3-O-Isopropylidenepyridoxine Hydrochloride (1) (13): About 2000 ml of acetone were distilled into a three-neck, 5-liter flask fitted with a drying tube. Pyridoxine hydrochloride (Sigma Chemical Company) (150 g) was added and the mixture was cooled to 0° to -10°C, saturated with gaseous hydrogen chloride (about 280 g) and stirred in the cold. The reaction was monitored by following the decrease in the absorption maximum at 325 nm at pH 7. Since the reaction was not complete after 48 hours, another 40 g of hydrogen chloride was added and stirring continued overnight in the cold. When ultraviolet monitoring indicated the reaction had reached completion, 500 ml of cold ether was added. The precipitate was filtered and dried yielding 172 g (96%). MP 208-210°C (13).

Isopropylidene-5-Pyridoxic Acid Hydrochloride (2): Potassium permanganate (97 g, 0.61 mol) dissolved in a minimum volume of water was stirred with 1 (88.3 g, 0.34 mol) dissolved in a minimal volume of water. The pH was approximately 5.5. After 10 min sodium hydroxide was added to bring the

pH to 7.3 and stirring was continued for 15 min. The manganese dioxide was filtered and washed with water. A second batch using 92 g (0.58 mol) potassium permanganate and 1 (83.7 g, 0.32 mol) was prepared in a similar manner. The filtrates and washings were combined and concentrated in vacuo. Concentrated hydrochloric acid was added to lower the pH to 5.3 where precipitation occurred. The solid was filtered and dried yielding 111.5 g (62%). MP 216-218°C (19).

3-Hydroxy-4-(Hydroxymethyl)-5-(Hydroxymethyl-d_2)-2-Methylpyridine Hydrochloride (Pyridoxine-d_2 Hydrochloride) (3): A suspension of 55.5 g (0.21 mol) (2) in 500 ml tetrahydrofuran (distilled over lithium aluminum hydride and ferrous sulfate) was mixed with a suspension of 10 g (0.24 mol) lithium aluminum deuteride in 2 L of tetrahydrofuran and stirred overnight at room temperature. The reaction was monitored by the shift in the absorption maximum at pH 7 from 281 nm for the acid to 290 nm for the alcohol. Water (about 55 ml) was added carefully to destroy excess deuteride. The precipitated hydroxides were filtered and the filtrate concentrated to yield 41.2 g (77%) of isopropylidene pyridoxine-d_2 hydrochloride. The isopropylidene group was removed by suspending the material in 0.1 N hydrochloric acid and heating on a steam bath for at least 1 h. The hydrolysis was monitored by following the increase in the absorption maximum at 325 nm at pH 7. Crystallization occurred upon cooling and additional product was obtained after further concentration. Total yield was 34 g (100% for the hydrolysis step). MP 210-212°C (20).

Isotopic composition: d_0 3.6%; d 10.7%; d_2 85.6%; d_3 0.2%.

Pyridoxine-d_2 (4): Ten grams (0.05 mol) 3 was added with stirring to 60 ml absolute ethanol containing 2 g (.05 mol)sodium hydroxide. The precipitated sodium chloride was extracted three times with 60 ml absolute ethanol. The combined extracts were evaporated in vacuo yielding 6.5 g (79%).

N-Benzyl-Pyridoxine-d_2 Bromide (5): 6.5 g (0.04 mol) (4) was dissolved in 80 ml methanol and added to a one liter flask containing 400 ml hot benzene. Benzyl bromide (20 ml, 0.17 mol) was added and the mixture refluxed for 16 h. The solution was concentrated in vacuo. The resulting precipitate was filtered and washed with hot ethanol yielding 5.5 g (42%) N-benzyl-Pyridoxine-d_2. MP 190°C. λ_{max} at 260 nm and 330 nm in 0.02 phosphate buffer pH 7.0.

3-Hydroxy-4-(Hydroxymethyl)-5-(Hydroxymethyl-d_2)-2-(Methyl-d_3)-Pyridine Hydrochloride (Pyridoxine-d_5-Hydrochloride) (6): Five and one half grams (0.016 mol) 5 was dissolved in 80 ml deuterium oxide plus 5 ml 40% sodium deuteroxide. The exchange was monitored by observing the loss of the 2-methyl proton signal at -140 Hz using proton magnetic resonance spectroscopy. Within 30 min the exchange was complete. The solution was neutralized with 10 N deuterium chloride, diluted to 200 ml with water and transferred to a hydrogenation vessel containing 300 mg catalyst (10% palladium on charcoal). The solution was then hydrogenated with shaking at 2.5 atm for 36 h. After filtering the catalyst the solution was evaporated to dryness in vacuo. The residue was extracted several times with ethanol. The washings were concentrated until a mixture of pyridoxine-d_5 hydrochloride and hydrobromide crystallized yielding 560 mg. Further concentration yielded an additional 375 mg for a total of 935 mg (28%). The mixture was converted completely to the hydrochloride form by dissolving 625 mg containing 2.6 mmol pyridoxine-d_5 in 5 ml water, passing the solution through a 5 ml column of Dowex 2-X8 (200-400 mesh, chloride form) and washing the column with about 15 ml water. The total eluate was lyophilized yielding 554 mg (100%) pyridoxine-d_5 hydrochloride. MP 203-204°C (20).

Isotopic composition: d_0 0.8%; d_1 1.7%; d_2 4.2%; d_3 3.6%; d_4 18.7%; d_5 70.2%; d_6 0.9%.

3-Hydroxy-5-(Hydroxymethyl-d_2)-2-Methyl-4-Pyridinecarboxylic Acid (7) (21): Three grams (0.014 mol) 3 was added to 300 ml of ethanol containing 3 g potassium hydroxide. Activated manganese dioxide (19 g) (22) was added and the suspension stirred at room temperature for 4 h. The manganous salts were oxidized by adding 10 ml 30% hydrogen peroxide. The solution was filtered immediately and the residue was washed with hot 0.1 M alcoholic potassium hydroxide. The combined filtrates were adjusted to pH 7 with concentrated hydrochloric acid and a small amount of manganese dioxide was filtered. Lowering the pH to 4 precipitated 4-pyridoxic acid-d_2 which was filtered after standing overnight in the cold yielding 0.975 g. Further concentration yielded an additional 135 mg. Total yield was 1.11 g (42%). MP 248-249°C (20).

Isotopic composition: d_0 8.9%; d_1 11.4%; d_2 79.7%; d_3 0.0%.

3-Hydroxy-5-(Hydroxymethyl-d_2)-2-Methyl-4-Pyridinecarboxaldehyde (Pyridoxal-d_2) (8): Five grams (0.024 mol) 3 was dissolved in 180 ml water with 1.57 ml concentrated sulfuric acid added. The solution was shaken vigorously with 16 g activated manganese dioxide for 75 s and immediately filtered using suction. The solution was concentrated to 40 ml, 15 g sodium acetate were added to adjust the pH to 5.6 and concentration was continued until crystallization began. Crystals were collected and the supernatant was concentrated to 10 ml to obtain more crystals. The combined crystalline product was washed with cold 95% ethanol and cold water. This product (5.29 g) was recrystallized twice by dissolving in water at pH 3.0 and then adjusting to pH 6.5. The crystallized pyridoxal was washed twice with cold water. Yield was 1.05 g (26%). A portion (999 mg) was dissolved in water, treated with the calculated amount of hydrochloric acid and crystallized by adding acetone yielding 1.10 g (91%) pyridoxal hydrochloride. MP 173-175°C (20).

Isotopic composition: d_0 3.5%; d_1 7.6%; d_2 88.7%; d_3 0.2%.

Pyridoxal-d_2 Oxime Hydrochloride (9) (23): 5.2 g (0.025 mol) 3 in 75 ml water was added with stirring 2.5 g (0.029 mol) activated manganese dioxide followed by dropwise addition of 1.4 ml (0.26 mol) concentrated sulfuric acid. The suspension was then heated to 60°-70°C in a water bath until the pH rose to about 6 and all of the manganese dioxide dissolved. Upon addition of 6.2 g sodium acetate and 2.6 g (0.079 mol) hydroxylamine hydrochloride a precipitate formed. After heating on a steam bath for 10 m the suspension was cooled in ice. The oxime hydrochloride was filtered, washed with water and dried yielding 3.2 g (58%). MP 220°C (20).

3-(Hydroxymethyl-d_2-4-(Aminomethyl)-5-Hydroxy-6-Methylpyridine Monohydrochloride (Pyridoxamine-d_2) (10): Absolute ethanol (200 ml) containing 2 g 9 was placed in a hydrogenation vessel with 1.5 g catalyst (10% palladium on carbon) and hydrogenated at 2.8 atm overnight. The catalyst was removed by filtration and the filtrate concentrated in vacuo until crystallization started. After standing in the cold overnight, the product was isolated yielding 1.06 g (56%). MP 225-226°C (20).

Isotopic composition: d_0 3.8%; d_1 11.3%; d_2 84.7%; d_3 0.2%.

Pyridoxamine-d_2-5'-Phosphate (11) : To a solution of 38.8 g phosphorus pentoxide in 30 ml 85% phosphoric acid at 60°C were added 6.4 g 10. The mixture was stirred at 55-65°C for 3 h and cooled. Then 3 ml of water, 160 ml ethanol and 500 ml ethyl ether were added and stirred. The solid product was filtered by suction and washed with ether. The fluffy white solid became brown and moist after standing overnight. This was dissolved in 320 ml 1.0 N HCl and heated for 30 min on a steam bath. The cooled solution was treated with 20 g Lloyds reagent (Harleco) for 15 min, cooled, filtered, and treated again with 10 g Lloyds reagent. The combined Lloyds reagent cakes were eluted with 500 ml 1.0 N NH_4OH. The extract was concentrated to 17 ml and adjusted to pH 5.7. This solution was applied to an Amberlite CG-50 column (H^+ form) (100-200 mesh, 98 x 1.7 cm) and eluted with water at 10-15 ml/h. Fractions between 620-1100 ml containing pure pyridox-

amine phosphate were collected and evaporated to dryness. The light tan solid was washed with cold water, ethanol and ether. Yield was 1.222 g (16%). A portion of the product (1.09 g) was dissolved in 17.5 ml hot water and recrystallized by addition of ethanol yielding 1.05 g. MP 238-240°C.

The low yield given for pyridoxamine phosphate is misleading. Most of the product eluted in earlier impure fractions. These fractions were not purified further because they were used to synthesize pyridoxal phosphate. If pyridoxamine phosphate were the only compound of interest, the product in the earlier fractions could be isolated to increase the yield. Peterson and Sober (18) commented on the hygroscopic nature of pyridoxamine phosphate. They calculated their preparation to be the dihydrate. Ours appeared to be the trihydrate. Pyridoxal phosphate also crystallized in the hydrated form. Because of the susceptibility of these derivatives to hydrolysis upon heating, they were analyzed in the hydrated form.

Pyridoxal-d_2-5'-Phosphate (12): The impure fractions eluting between 100-620 ml from the Amberlite CG-50 column used for pyridoxamine phosphate purification were combined with the washings from the purified pyridoxamine phosphate. The total volume was made to 800 ml. The solution was stirred vigorously while 5.0 g active manganese dioxide were added. The reaction was monitored by ultraviolet absorption measurements at 388 nm and was stopped after two hours when the reading had stabilized. The burgundy-brown solution was filtered using suction, adjusted to pH 7.0 with 5 N NaOH, concentrated to 50 ml, and refrigerated overnight. The resultant brown precipitate was centrifuged and discarded. The supernatant was concentrated to 35 ml and applied to an Amberlite CG-50 column (H+ form) (98 x 1.7 cm) and eluted with water at 15 ml/h. The yellow fractions from 220-280 ml were combined, concentrated to 5 ml, and applied to an Amberlite CG-120 column (H+ form) (200-400 mesh, 87 x 1.7 cm) and eluted with water at 15-20 ml/h. The yellow fractions from 1350-1650 ml were combined and concentrated to

3 ml. Overnight refrigeration crystallized the yellow solid, which was filtered and dried in vacuo. Yield was 1.186 g (approximately 40%). A portion of the product (1.101 g) was dissolved in a minimal amount of water and recrystallized by adding acetone yielding 895 mg (81.3%). MP 142-144°C.

Pyridoxine-d_2-5'-Phosphate (13): Isopropylidene pyridoxine-d_2 hydrochloride (5.9 g) was made as described previously from 5.1 g 3. This entire amount was added to a mixture of 21.2 ml 85% phosphoric acid and 26.5 g phosphorus pentoxide, which had been stirring previously 2 h, and stirred for six hours at 50-60°C. The mixture was allowed to cool and stand at room temperature overnight. Water (100 ml) was added slowly and the mixture was stirred for two hours at 85-90°C, then for two hours at room temperature. The rose colored solution was treated by stirring with 20 g Lloyds reagent for 15 min. The Lloyds reagent was removed by filtration and the solution was treated twice more in the same manner. The combined solid cakes (60 g) were extracted by stirring for twenty minutes with 600 ml 1.0N NH_4OH.

The extract was concentrated to 20 ml and the brown oil was adjusted to pH 5.8 and applied to a Dowex 50 column (H+ form) (28 x 1.5 cm). The column was washed with water and the fractions 130-800 ml were collected and combined. The combined effluent, containing pyridoxine phosphate and another product visible under ultraviolet light, was concentrated to 70 ml and refrigerated overnight. A small amount of precipitate was centrifuged and discarded. The supernatant was concentrated to 8 ml and the suspension was adjusted to pH 5.3 to dissolve all material. This solution was applied to an Amberlite CG-120 column (H+ form) (200-400 mesh, 87 x 1.7 cm). The column was eluted with water at 15-20 ml per hour and the fractions between 1900-2900 ml containing pure pyridoxine phosphate were collected. The combined fractions were evaporated to leave a white powder. The yield was 1.348 g (22%). A portion of this (1.264 g) was recrystallized from 10 ml hot water yielding 1.03 g (82%). MP 201-203°C.

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