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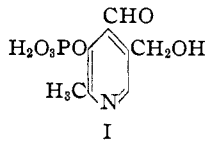
Phosphates of the Vitamin B_6 Group. II. 3-Pyridoxalphosphoric Acid

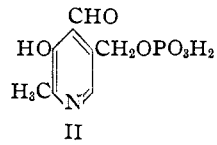
BY DOROTHEA HEYL AND STANTON A. HARRIS

Several groups, including a phosphoric acid group, have been substituted on the 3-hydroxyl group of pyridoxal. 3-Pyridoxalphosphoric acid has been characterized as an oxime which differs from the oxime of codecarboxylase.

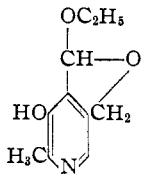
Derivatives of 3-pyridoxalphosphoric acid (I) have been synthesized, and have been used to corroborate the evidence[1,2,3] that 3-pyridoxalphosphoric acid is different from codecarboxylase, 2 - methyl - 3 - hydroxy - 4 - formyl - 5 - pyridylmethylphosphoric acid (II).



I



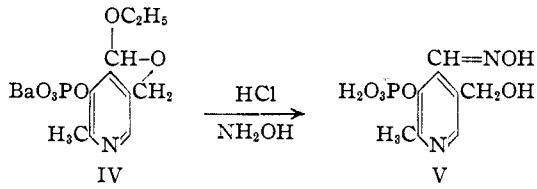
II



III

The phosphorylation of pyridoxal monoethyl acetal (III)[4] at the 3-hydroxyl group in pyridine solution with phosphorus oxychloride was reported previously.[5] This phosphorylation was also carried out by Karrer and Viscontini.[6]

The monoethyl acetal of 3-pyridoxalphosphoric acid was isolated as the crystalline barium salt (IV). This monoethyl acetal was hydrolyzed by

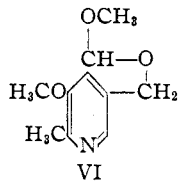


IV V

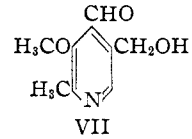
reaction with hydrochloric acid in the presence of hydroxylamine hydrochloride. The oxime of 3-pyridoxalphosphoric acid (V) crystallized from the solution; the melting point, 210–211°, of this oxime was not in agreement with the melting point of codecarboxylase oxime,[1] 229–230°, and the melting point of a mixture of the oximes showed a depression. The absorption spectrum[7] of this oxime also differed from that of codecarboxylase oxime (Figs. 1 and 2). No ferric chloride color test was observed with this oxime, whereas codecarboxylase oxime gives a ferric chloride test. Finally, 3-pyridoxalphosphoric acid had no codecarboxylase activity.[2,3]

(1) Heyl, Luz, Harris and Folkers, THIS JOURNAL, **73**, 3430 (1951).
(2) Gunsalus and Umbreit, *J. Biol. Chem.*, **170**, 415 (1947).
(3) Umbreit and Gunsalus, *ibid.*, **179**, 279 (1949).
(4) Harris, Heyl and Folkers, THIS JOURNAL, **66**, 2088 (1944).
(5) Heyl, Harris and Folkers, Abstracts, American Chemical Society, 110th Meeting, Chicago, 35B (1946).
(6) Karrer and Viscontini, *Helv. Chim. Acta*, **30**, 52 (1947).
(7) We are indebted to Dr. Charles Rosenblum and his associates for the ultraviolet absorption measurements.

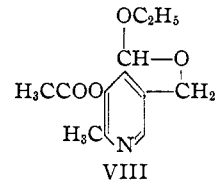
The monomethyl acetal of 3-pyridoxal methyl ether (VI) was prepared by the action of diazomethane on pyridoxal monomethyl acetal.[1] Hydrolysis of the acetal to form 3-pyridoxal methyl ether (VII) requires more vigorous conditions than does the hydrolysis of pyridoxal monoethyl acetal.[4] The absorption spectra of compounds IV, VI and VII, all having substituents on the 3-hydroxyl group, are similar to each other (Figs. 3 and 4).



VI



VII



VIII

The monoethyl acetal of 3-pyridoxal acetate (VIII) was also prepared. All attempts to selectively hydrolyze the acetal group resulted in complete hydrolysis to pyridoxal. This hydrolysis has also been observed by Karrer, Viscontini and Forster.[8]

Experimental[9]

Barium Salt of the Monoethyl Acetal of 3-Pyridoxalphosphoric Acid (IV).—The monoethyl acetal of pyridoxal hydrochloride (1.7 g.) dissolved in 50 ml. of dry pyridine, protected from moisture and cooled to −20° in a Dry Ice–carbon tetrachloride-bath, was treated with 0.8 ml. of phosphorus oxychloride in small portions. The temperature did not rise above −15°. When the phosphorus oxychloride had been added, the cooling bath was removed. The temperature rose gradually to room temperature, and the mixture was cooled again to −20°. Ten cubic milliliters of water was added in small portions, accompanied by cooling so that the solution temperature did not exceed 0°. The cooling bath was again removed, and when the temperature had risen to room temperature, 10 ml. of water was added. The solution was concentrated to a sirup under reduced pressure with the use of a bath not exceeding 50°. The sirup was again cooled in the Dry Ice-bath and neutralized to litmus with barium hydroxide solution and solid barium hydroxide. The thick precipitate was removed by centrifuging and was washed several times with water. The resulting solutions were combined and concentrated as before. The concentrate, cooled well below 0° and kept at that temperature, was made acid to Congo red with 6 *N* sulfuric acid. After removal of barium sulfate by centrifuging, solid silver carbonate was added until the chloride ion was completely removed. The excess silver ion was removed by means of hydrogen sulfide. The filtrate was made alkaline with barium hydroxide, neutralized with carbon dioxide, centrifuged and concentrated. The addition of two volumes of ethyl alcohol caused the precipitation of the crystalline barium salt of the monoethyl acetal of 3-pyridoxalphosphoric acid. It was recrystallized by solution in water and reprecipitation with alcohol. After centrifuging it was washed with alcohol, then ether and dried.

Anal. Calcd. for $C_{10}H_{12}NO_6PBa$: N, 3.41. Found: N, 3.58.

Oxime of 3-Pyridoxalphosphoric Acid (V).—One-half gram of the barium salt of the monoethyl acetal of 3-pyri-

(8) Karrer, Viscontini and Forster, *Helv. Chim. Acta*, **31**, 1004 (1948).
(9) We are indebted to Mr. Richard Boos and his associates for the microanalyses.

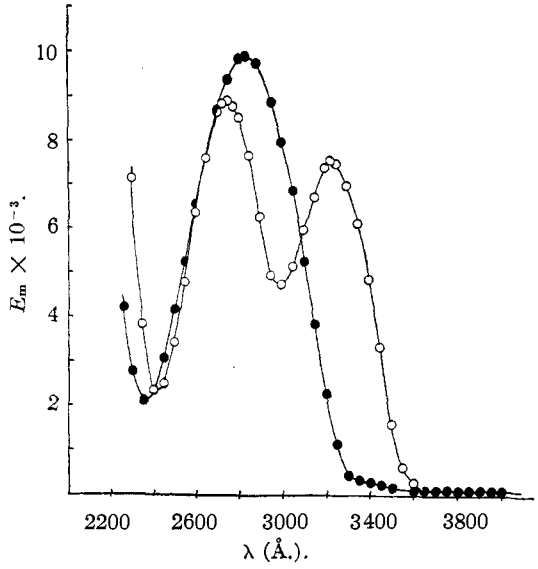


Fig. 1.—Absorption spectra at *p*H 2: O, codecarboxylase oxime; ●, oxime of 3-pyridoxalphosphoric acid (V).

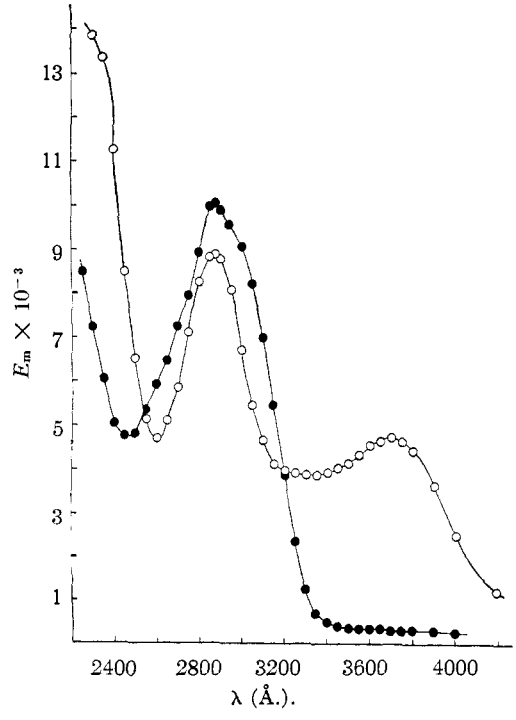


Fig. 2.—Absorption spectra at *p*H 11.0: O, codecarboxylase oxime; ●, oxime of 3-pyridoxalphosphoric acid (V).

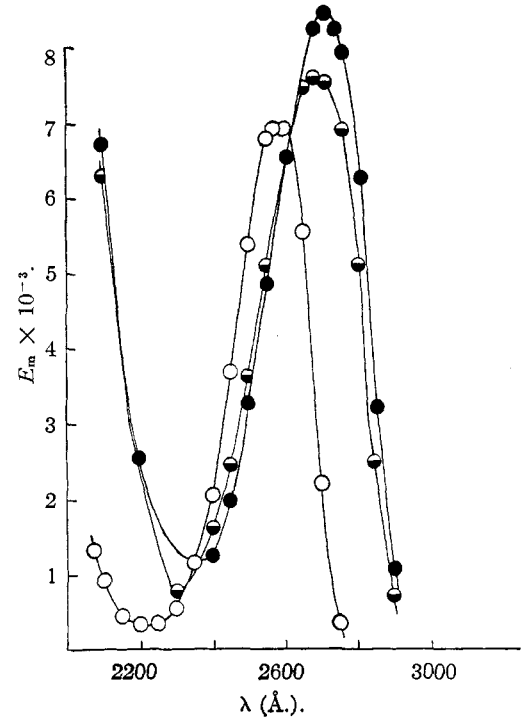


Fig. 3.—Absorption spectra at *p*H 1.9: O, monoethyl acetal of 3-pyridoxalphosphoric acid (IV); ●, monomethyl acetal of 3-pyridoxal methyl ether (VI); ◒, 3-pyridoxal methyl ether (VII).

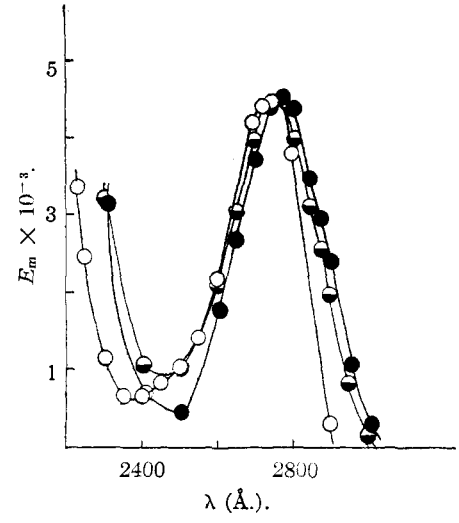


Fig. 4.—Absorption spectra at *p*H 10.9: O, monoethyl acetal of 3-pyridoxalphosphoric acid (IV); ●, monomethyl acetal of 3-pyridoxal methyl ether (VI); ◒, 3-pyridoxal methyl ether (VII).

doxalphosphoric acid dissolved in 3 ml. of water was treated with 0.1 g. of hydroxylamine hydrochloride. The solution was acidified to *p*H 1–2 with 6 *N* hydrochloric acid. After two minutes warming on the steam-bath, the solution was cooled in ice. Crystals of the oxime of 3-pyridoxalphosphoric acid separated quickly; yield 107 mg. (34%); m.p. 210–211° (dec.).

Anal. Calcd. for $C_8H_{11}N_2O_6P$: C, 36.64; H, 4.23; N, 10.69; P, 11.83. Found: C, 36.58; H, 4.04; N, 10.99; P, 11.80.

Monomethyl Acetal of 3-Pyridoxal Methyl Ether (VI).—In a solution of 1 ml. of concentrated alcoholic hydrogen chloride in 200 ml. of methyl alcohol, 20.0 g. of pyridoxal hydrochloride was suspended. After this mixture had been warmed on the steam-bath for a few minutes and had stood at room temperature for another half-hour, 13 g. of sodium bicarbonate was added in several portions, and the suspension was refluxed for an hour. When the solution had been filtered to remove inorganic material, it was cooled in an ice-bath, and diazomethane in ether was added in small portions until no more reacted. After another filtration to remove inorganic material, the solution was concentrated to dryness under reduced pressure, and the oily residue was shaken with ether several times. The ether extracts were decanted, washed four times with water, decolorized with Darco, concentrated to dryness under reduced pressure, and the residue dried by distillation with benzene. The monomethyl acetal of 3-pyridoxal methyl ether crystallized from petroleum ether (b.p. 30–60°).

yield 6.71 g. (35%); m.p. 54–55°, which did not change on recrystallization.

Anal. Calcd. for $C_{10}H_{13}NO_3$: C, 61.52; H, 6.71; N, 7.18. Found: C, 61.77; H, 6.51; N, 7.24.

3-Pyridoxal Methyl Ether (VII).—A solution of 4.68 g. of the monomethyl acetal of 3-pyridoxal methyl ether in water was adjusted to *p*H 2 with hydrochloric acid. After brief warming and subsequent standing at room temperature for three-quarters of an hour, the solution was concentrated almost to dryness, and acetone and ether were added. Crystals of the hydrochloride of the monomethyl acetal of 3-pyridoxal methyl ether crystallized in a yield of 3.72 g. (67%); m.p. 152–153°.

Anal. Calcd. for $C_{10}H_{14}NO_3Cl$: C, 51.84; H, 6.09; N, 6.05. Found: C, 52.03; H, 6.00; N, 6.04.

Under the conditions of this experiment, pyridoxal monoethyl acetal is completely hydrolyzed to pyridoxal.[4]

On neutralization of an aqueous solution of the hydrochloride with sodium bicarbonate, subsequent distillation to dryness, and extraction of the residue with petroleum ether, crystals of the monomethyl acetal of 3-pyridoxal methyl ether melting at 53–54° were obtained. The melting point of a mixed sample of these crystals and the material from which the hydrochloride was made, was the same.

The hydrolysis was repeated under more vigorous conditions. A solution of 1.0 g. of the monomethyl acetal of 3-pyridoxal methyl ether hydrochloride in 15 ml. of water was treated with 3.5 ml. of 6 *N* hydrochloric acid. After 30 minutes on the steam-bath and two days at room temperature, the solution was distilled almost to dryness under reduced pressure. The crystalline 3-pyridoxal methyl ether hydrochloride was suspended in acetone and then filtered; yield 0.81 g. (86%); dec. 179–180°. This decomposition point was not changed on recrystallization from water–acetone.

Anal. Calcd. for $C_9H_{12}NO_3Cl$: C, 49.66; H, 5.56; N, 6.44. Found: C, 50.14; H, 5.88; N, 6.64.

Monoethyl Acetal of 3-Pyridoxal Acetate Hydrochloride (VIII).—Five milliliters of acetic anhydride containing 0.23 g. of the monoethyl acetal of pyridoxal hydrochloride and two drops of alcoholic hydrogen chloride was stirred for two hours at approximately 50°. The resulting homogeneous solution was "freeze-dried." After a little alcohol had been added to the residue and the volatile material removed under reduced pressure, the residue was crystallized from alcohol–ether; yield 0.22 g. (82%). After recrystallization from alcohol–ether, the monoethyl acetal of 3-pyridoxal acetate hydrochloride melted at 161–162°. It gave no color reaction with ferric chloride solution.

Anal. Calcd. for $C_{12}H_{16}NO_4Cl$: C, 52.65; H, 5.89; N, 5.12. Found: C, 52.84; H, 5.79; N, 5.58.

RAHWAY, N. J. RECEIVED JANUARY 12, 1951

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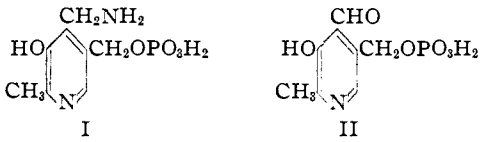
Phosphates of the Vitamin B_6 Group. III. Pyridoxamine Phosphate

BY DOROTHEA HEYL, EILEEN LUZ, STANTON A. HARRIS AND KARL FOLKERS

Pyridoxamine phosphate (2-methyl-3-hydroxy-4-aminomethyl-5-pyridylmethylphosphoric acid) has been synthesized, isolated as an amorphous calcium salt, and characterized as a crystalline di-*p*-toluenesulfonyl derivative. Attempts to obtain pyridoxamine phosphate by catalytic hydrogenation of codecarboxylase oxime have resulted in removal of the phosphate group.

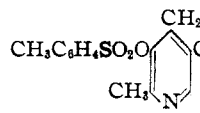
Pyridoxamine phosphate (I) has been synthesized and characterized as a crystalline di-*p*-toluenesulfonyl derivative. This phosphate (I) is the pyridoxamine analog of codecarboxylase (II).[1]



I II

It has been shown that pyridoxamine phosphate is a growth factor for lactic acid bacteria.[2,3] Since pyridoxamine phosphate was prepared[2] "in solution" (not isolated) by autoclaving codecarboxylase with glutamic acid, the phosphoric acid group must be in the same position in pyridoxamine phosphate as in codecarboxylase.

Pyridoxamine phosphate has now been prepared by direct phosphorylation of pyridoxamine in aqueous solution with phosphorus oxychloride, and has been isolated as a crude calcium salt. Since this material[2] has the same activity as that[2] prepared by amination of the aldehyde, the free acid has structure I. On reaction with *p*-toluenesulfonyl chloride in the presence of sodium hydroxide, and subsequent acidification, 2-methyl-3-*p*-toluenesulfonoxy - 4 - *p* - toluenesulfonylaminomethyl - 5-pyridylmethylphosphoric acid (III) was obtained in crystalline form and was satisfactorily characterized. This compound no longer gives color tests with ferric chloride or diazotized aniline, as do pyridoxamine and pyridoxamine phosphate. Therefore, one *p*-toluenesulfonyl group is on the 3-hydroxy group; the second is on the primary amino group.



III, R = PO_3H_2
IV, R = H

A similar di-*p*-toluenesulfonyl (IV) derivative was prepared from pyridoxamine by the same method.

The hydrogenation of pyridoxal oxime to pyridoxamine has been described previously.[4] Attempts to convert codecarboxylase oxime to pyridoxamine phosphate by hydrogenation over palladium or nickel catalysts have resulted in removal of the phosphoric acid group.

Experimental[5]

Calcium Pyridoxamine Phosphate (Calcium Salt of 2-Methyl-3-hydroxy-4-aminomethyl-5-pyridylmethylphosphoric Acid (I)).—A solution of 5 g. of pyridoxamine dihydrochloride in 35 ml. of water was surrounded by a cold water-bath. The solution was stirred mechanically while 25 ml. of phosphorus oxychloride was added at such a rate that the temperature did not exceed 50°; the addition, which required 75 minutes, was followed by 30 minutes of stirring. After as much hydrogen chloride as possible had been removed *in vacuo*, the solution was surrounded by an

(1) Heyl, Luz, Harris and Folkers, THIS JOURNAL, **73**, 3434 (1951).
(2) McNutt and Snell, *J. Biol. Chem.*, **182**, 557 (1950).
(3) Hendlin, Caswell, Peters and Wood, *ibid.*, **186**, 647 (1950).
(4) Harris, Heyl and Folkers, THIS JOURNAL, **66**, 2088 (1944).
(5) We are indebted to Mr. Richard Boos and his associates for the microanalyses.