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Syntheses of Organic Phosphates. I. Pyridylmethyl Phosphates*,**

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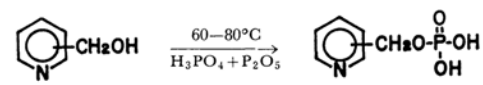
The reaction of pyrophosphoric acid with 2-, 3- and 4-pyridylmethanols resulted in the formation of the corresponding pyridylmethyl phosphates. The separation of each ester from the corresponding phosphorylation mixture was performed by passing the mixture through a column of Dowex 50W-X8 [H^+] resin. The 2-, 3-, and 4-isomers were recrystallized from ethanol-water as white crystals with melting points of 182—182.5°C (part. decomp.), 194.5—195.5°C (part. decopm.), and 201.5—202.5°C (decomp.) respectively. An attempt to separate each of these esters from an artificial mixture of them consisting of an equal amount of each was performed by using an ion-exchange resin, Dowex 50W-X8 [H^+]. The acid dissociation constants were determined by a potentiometric method at 25.0°C and $\mu=0.10$ M (KNO_3); this gave three pK's for each phosphate, pK_{H_3A}, pK_{H_2A}, and pK_{HA}: 2-pyridylmethyl phosphate, 1.8, 4.42, 6.29; 3-pyridylmethyl phosphate, 1.9, 4.86, 6.23; 4-pyridylmethyl phosphate, 1.7, 5.14, 6.25. Of these pK's, p$K_{H\ A}$ refers to the dissociation of a pyridinium proton, while the other pK's refer to those of phosphate protons.

In connection with the studies of nucleotides, various organic phosphates have been synthesized extensively, and many useful phosphorylating reagents have been developed as reviewed in the literature.[1-4] However, since the existing reagents have evident limitations, an appropriate phosphorylating reagent needs to be selected for a specified synthesis depending upon the structure and properties of the alcohol which is to undergo reaction.

In the present work, pyridylmethyl phosphates (I), the simplest analogs of vitamin B_6 phosphate, were prepared. The phosphorylation of 2-, 3-, and 4-pyridylmethanol by the use of pyrophosphoric acid, prepared from 85% orthophosphoric acid and phosphorus pentoxide, was performed with some modification, according to the procedures employed for the syntheses of the substituted 3-pyridylmethyl phosphates.[5] 3-Pyridylmethyl phosphate was also synthesized by the reaction of 3-chloromethylpyridine hydrochloride with trisilver phosphate in ether. The yield was not, however, satisfactory because of the heterogeneous nature of the reaction. Among the phosphates synthesized in this study, 3-pyridylmethyl phosphate had been prepared before and the vasodilating effect of its salts described.[6]



I

The Isolation of Phosphate Esters from Phosphorylation Mixtures.—The main problem in the present preparation was how to achieve the separation of the desired phosphates from phosphorylation mixtures which contain a large excess (about 6 to 12 times mol.) of inorganic phosphoric acid. A chromatographic method using cation-exchange resin, Dowex 50W-X8, was found most effective and simple for the separation of phosphate esters.

Some other methods of separation were also tried, however, for the sake of comparison. For 3- and 4-pyridylmethyl phosphates, the phosphates were separated as precipitates from coexisting inorganic phosphates when the reaction mixtures were treated with ethanol-ether. On the other hand, in the case of 2-pyridylmethyl phosphate, such a precipitation procedure failed to have any advantage. Only a small amount of the precipitate, which contains a fairly large amount of inorganic phosphate, was obtained from the reaction mixture. This precipitate was then dissolved in a small amount of water, and saturated aqueous barium hydroxide was added to adjust the final pH of the solution to about 11. Then the mixture was filtered to remove the white precipitate formed. The addition of two

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1) G. M. Kosolapoff, "Organophosphorus Compounds," John Wiley & Sons, New York (1950).

2) D. M. Brown, "Advances in Organic Chemistry," Vol. 3, Interscience Publishers, New York (1963), p. 75.

3) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley & Sons, New York (1961).

4) F. Cramer, *Angew. Chem.*, **72**, 236 (1960).

5) E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.*, **76**, 169 (1954).

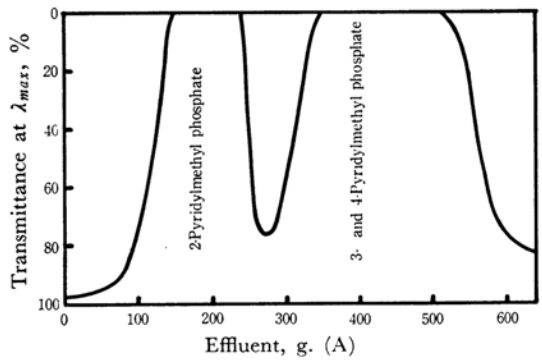
6) R. F. Long and A. L. Morrison, U. S. Pat. 2777851 (1957); *Chem. Abstr.*, **51**, 12984 (1957).

volumes of ethanol to the filtrate brought down the white precipitate of barium salt. The barium salt was purified by re-precipitation with ethanol from its aqueous solution. Although the phosphorus analysis showed this white solid to be nearly pure as barium 2-pyridylmethyl phosphate, $C_6H_6NO_4PBa$, the yield was poor not exceeding a few per cent. Two major factors may be responsible for this low yield: first, the precipitation of phosphate ester from the reaction mixture was not effected by the combination of ethanol and ether at the initial step; and second, the inorganic phosphate precipitated upon the addition of saturated barium hydroxide contained a fairly large fraction of an organic substance. An attempt has been made to separate 3-pyridylmethyl phosphate as its barium salt through a procedure similar to that mentioned above, but it resulted in a poor yield. The barium salt of an ester was converted into the corresponding free form, either by precipitating barium sulfate with sulfuric acid or by passing its water suspension through a column of Dowex 50W-X8 [H^+] resin.

On the other hand, for the isolation of 3-pyridylmethyl phosphate from the phosphorylation mixture, repeated precipitation with ethanol was proved to be effective, though less so than the chromatographic method using cation-exchange resin.

The Separation of Phosphate Esters from an Artificial Mixture.—The experimental conditions which were required for the isolation of each isomeric phosphate ester from the coexisting orthophosphoric acid were somewhat different from each other. Therefore, the separation procedure used for 2-pyridylmethyl phosphate was not applicable to the other isomers. In order to obtain some additional information about these separation procedures, an artificial mixture which contained equal amounts of the three esters in an aqueous solution was run onto an ion exchange column of Dowex 50W-X8 resin, both in the acid and in the ammonium forms. The eluting behavior is illustrated in Fig. 1. It may be seen that a satisfactory separation of 2-pyridylmethyl phosphate was achieved with the acid-form resin, while 3- and 4-isomers were not separated from each other even though the initial fractions of the mixed effluent were largely composed of the 3-isomer and the final fractions were composed of the 4-isomer, judging from their ultraviolet absorption spectra (Fig. 1-A). With the ammonium-form resin used, 2-pyridylmethyl phosphate failed to be recovered from the ion exchange column in this experiment (Fig. 1-B). It should be noted that the eluting behavior of 3- and 4-pyridylmethyl phosphate were considerably different from those in the presence of inorganic phosphoric acid, where these phosphates exhibited a retention time as short as that of inorganic phosphate and failed to be separated from the latter as a result.

Acid Dissociation Constants.—The three



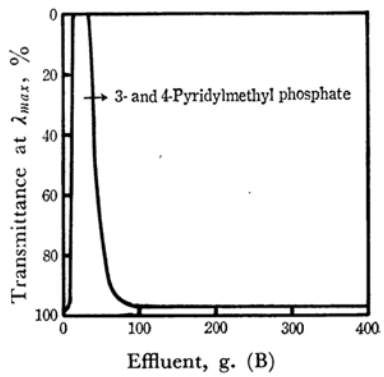


Fig. 1. Separation of phosphate esters from an artificial mixture by ion exchange chromatography.
Mixture: 20 mg. each of the three isomeric esters in 4 ml. aqueous solution.
Column: (A) Dowex 50W-X8 [H^+] (40 meq.), 1.5×15 cm.
(B) Dowex 50W-X8 [NH_4^+](40 meq.), 1.5×15 cm.
Eluent: mineral-free water.
Elution rate: 10 g./hr.

TABLE I. ACID DISSOCIATION CONSTANTS OF PYRIDYLMETHYL PHOSPHATES

25.0±0.1°C, μ=0.10 M (KNO_3)

Acid	pK_{H_3A}	pK_{H_2A}	pK_{HA}
2-Pyridylmethyl phosphate	1.8	4.42	6.29
3-Pyridylmethyl phosphate	1.9	4.86	6.23
4-Pyridylmethyl phosphate	1.7	5.14	6.25

isomeric pyridylmethyl phosphates prepared in this work were proved to be tribasic acids, as illustrated by the three kinds of acid dissociation constants in Table I. The value of K_{H_3A} and that of K_{HA} do not vary significantly among the three isomers, while the K_{H_2A} value changes from one to the other. In addition, these pK_{H_3A} and pK_{HA} values are within a range of magnitude of the acid dissociation constants for ordinary dibasic phosphate monoesters.[7] Therefore, those protons dissociating in the first and third processes are most likely to be attributed to the phosphate-protons, while

the second dissociation step seems to involve a pyridinium proton, as is shown in Chart 1.* The

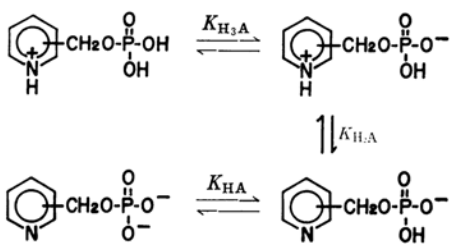


Chart. 1 Acid dissociation processes for pyridylmethyl phosphates.

pK_{H_2A} value is seen to be smallest with the methylphosphate group at the 2-position, and it increases as the substituent is placed further from the 1-position, the largest value being obtained with the 4-isomer. The electron-withdrawing effect of the substituent may provide a reasonable basis for the variation in the acidity of a pyridinium proton. An additional effect would be generated through the participation of the phosphate group in the formation of hydrogen bonding with pyridine-nitrogen in the case of 2-pyridylmethyl phosphate. In other words, such a tendency of hydrogen bond formation would facilitate the liberation of a pyridinium proton.

Experimental

2-Pyridylmethanol.—This material was synthesized from 2-picoline through the rearrangement of 2-picoline *N*-oxide to the acetate of 2-pyridylmethanol, which was subsequently hydrolyzed by acid to the methanol according to the method of Boekelheide and Linn;[8] b. p. 114—116°C/16—17 mmHg (reported[8] b. p. 110—112°C/15 mmHg); the picrate, m. p. 160.5—162.0°C (reported[8] m. p. 160.5—161.0°C).

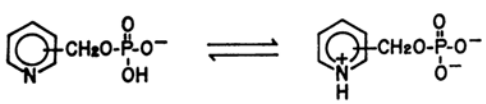
3-Pyridylmethanol.—This alcohol was prepared by the reduction of ethylnicotinate (purchased from the Tokyo Kasei Kogyo Co. Ltd., Tokyo) with lithium aluminum hydride;[9-11] b. p. 117.5—118°C/3 mmHg (reported b. p. 144—145°C/20 mmHg,[9] 144°C/10 mmHg[10]); the picrate, m. p. 162.2—162.7°C (reported m. p. 159°C,[10] 158—160°C[11]).

4-Pyridylmethanol.—This methanol was synthesized from 4-picoline through a preparative route similar to that used for 2-pyridylmethanol, though the procedures were slightly modified from those mentioned in the literature.[8,12] 4-Picoline *N*-oxide was allowed to react with acetic anhydride at approximately 45°C for 2 hr., and then the reaction temperature was gradually raised to 90°C in 4 hr. After the mixture had been refluxed for a few minutes, the acetate of 4-pyridylmethanol containing an appreciable amount of acetic anhydride was distilled off. This acetate fraction with a boiling range of 99—107°C at 6 mmHg was hydrolyzed with 5 N hydrochloric acid, and then concentrated to dryness in vacuo. The residue was neutralized with aqueous potassium carbonate, made strongly alkaline with 20% sodium hydroxide, and then extracted with chloroform and ether. The combined extracts dried over anhydrous potassium carbonate were distilled to give the product; b. p. 124—128°C at 6 mmHg (reported b. p. 107—110°C/1 mmHg,[13] 152—154°C/15 mmHg[14]); m. p. 51—55°C (reported m. p. 57—60°C,[8] 57.8—58.8°C[12]); the picrate, m. p. 156.5—158°C in a sealed tube (reported m. p. 157—161°C[13]); yield 13 g. (14% from *N*-oxide).

2-Pyridylmethyl Phosphate.—A clear viscous liquid was obtained by the brief heating of 85% orthophosphoric acid and of phosphorus pentoxide at a 1 : 1 weight ratio.[15] A 20.2 g. sample of this mixture was mixed with 3.7 g. of 2-pyridylmethanol at room temperature in a 50-ml. flask protected from moisture. This reaction mixture, after having been heated on a water-bath at 77°C for 10 hr. with frequent shaking, was diluted with 10 ml. of water and then heated at 90°C for 30 min. in order to hydrolyze any polyphosphoric compounds. Approximately an equal volume of water was added to the hydrolysate, which was then divided into two portions, each applied to the top of a column (2×28 cm.) of Dowex 50W-X8 [H^+] resin (130 meq.) and eluted with water at 30 g. per hour. After the front effluent, which was strongly acidic (pH≈0.4), came off, the acidity of the effluent gradually lowered, reaching pH 4.0 at a total effluent of 100 ml. The collection of the fractions of the next 100-ml. portion resulted in the further pH elevation at the end to 4.8, which is nearly the same as that of the eluent (water). The ultraviolet measurements of the eluted fractions collected up to this stage showed the presence of no, or only trace amounts of, aromatic species. Then, an effluent turned acidic, accompanied by a strong increase in the optical density. After 450 ml. of the subsequent effluent had been collected, the pH came back to 4.8—5.0 and the ultraviolet absorption at 261 mμ disappeared. These latter fractions (450 ml.), which contained the phosphate ester, were concentrated to about 10 ml. under reduced pressure below 40°C. Tiny, white, pillar-like crystals were precipitated by adding 100 ml. of ethanol and by leaving the solution in a refrigerator overnight. After filtration, the precipitate was washed with ethanol; yield 5.5 g. (85%); m. p. 181—181.5°C (part. decomp.); upon recrystallization from ethanol-water (4 : 1), m. p. 182—182.5°C (part. decomp); extremely soluble in water and insoluble in ethanol; neutralization equivalence 94.8 (calcd.

7) J. R. Van Wazer, "Phosphorus and Its Compounds," Vol. I, Interscience Publishers, New York (1958), p. 583.

* These dissociation processes do not exclude the possibillity of equilibria existing within each ionic species illustrated in Chart I; i. e., for the monoionic species:



8) V. Boekelheide and W. J. Linn, *J. Am. Chem. Soc.*, **76**, 1286 (1954).

9) R. G. Jones and E. C. Kornfeld, ibid., **73**, 107 (1951).

10) K. W. Rosenmund and F. Zymalkoski, *Chem. Ber.*, **85**, 152 (1952).

11) F. Bohlmann and M. Bohlmann, ibid., **86**, 1419 (1953).

12) J. A. Berson and T. Cohen, *J. Am. Chem. Soc.*, **77**, 1281 (1955).

13) H. S. Mosher and J. E. Tessieri, ibid., **73**, 4925 (1951).

14) M. Protiva, *Chem. Listy*, **45**, 20 (1951); *Chem. Abstr.*, **45**, 8997 (1951).

15) J. E. Malowan, "Inorganic Syntheses," Vol. III, 96 (1950).

94.6); λ_{max} 261 mμ (10^{-4} M aqueous solution), ε 6200.

Found: C, 38.22; H, 4.42; N, 7.54; P, 16.0. Calcd. for $C_6H_8NO_4P$: C, 38.11; N, 4.26; H, 7.41; P, 16.4%.

A satisfactory separation was also attained by neutralizing the partially-hydrolyzed phosphorylation mixture with concentrated aqueous ammonia, followed by chromatographic separation with a column of Dowex 50W-X8 [H^+] resin. In this case, a larger amount of the ion exchanger was needed to convert the ammonium salt to the corresponding free acid, but the elution was carried out in the same manner, as is shown in Fig. 2.

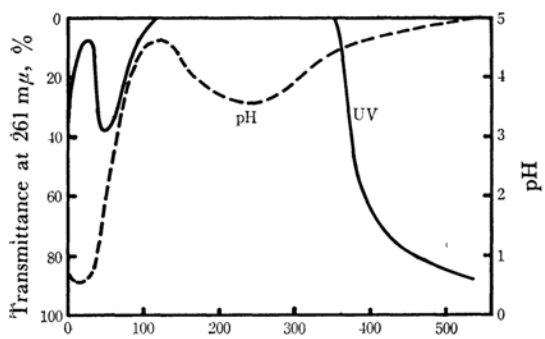


Fig. 2. Chromatographic separation of 2-pyridylmethyl phosphate from the phosphorylation mixture.
Mixture: partially-hydrolyzed phosphorylation mixture (with 0.4 g. of the alcohol as a starting material) neutralized to pH 8 with concentrated aqueous ammonia
Column: Dowex 50W-X8 [H^+] (130 meq.), 2×28 cm.
Eluent: mineral-free water
Elution rate: 30 g./hr.

3-Pyridylmethyl Phosphate.—The phosphorylation mixture, which was obtained by the reaction of 4 g. of 3-pyridylmethanol and 40 g. of pyrophosphoric acid in accordance with the method of Long and Morrison,[6] was poured into ethanol, and then ether was added. After the mixture had been kept in a refrigerator overnight, the clear supernatant liquid decanted. The residue dissolved in 70 ml. of 1 N hydrochloric acid and heated at 75°C for 30 min. To this acidic solution, after it had been concentrated to about 20 ml. on a water-bath (45°C) in vacuo, 100 ml. of hot acetone was added with vigorous shaking. After the mixture had then been cooled to room temperature, 100 ml. of ether was poured into it. The oily material which separated from the supernatant liquid was dissolved in a small volume of water and reprecipitated with ethanol. Three quarters* of the precipitate were neutralized to pH 8 with aqueous ammonia, then evaporated to dryness in vacuo. The residue, crystalline ammonium salt, was again dissolved in a minimum amount of water and charged to the top of a column (1.4×30 cm.) of Dowex 50W-X8 [H^+] resin (about 80 meq.). The elution was conducted with water at the rate of 20 g. per hour. As the pH of the effluent rose rather steadily throughout the elution, from strongly acidic to neutral without any distinctive features for separation, the effluent fractions were divided into two parts according to the optical density around 260 mμ. The second part consisted of fractions which came off beyond pH 2.0; its total volume (about 500 ml.) was concentrated to about 5 ml. in vacuo below 40°C. A white crystalline precipitate was recovered from the concentrated solution by adding 50 ml. of ethanol; it was recrystallized from ethanol-water (4 : 1) as tiny white pillars; yield 2.4 g. (50%); m. p. 194.5—195.5°C (part. decomp.) (reported m. p. 195—196°C[6]); extremely soluble in water and insoluble in ethanol; neutralization equivalence 94.3 (calcd. 94.6); λ_{max} 260 mμ (10^{-4} M aqueous solution), ε 5100.

Found: C, 38.17; H, 4.64; N, 7.34; P, 17.1. Calcd. for $C_6H_8NO_4P$: C, 38.11; H, 4.26; N, 7.41; P, 16.4%.

4-Pyridylmethyl Phosphate.—The phosphorylation of 4-pyridylmethanol (4 g.) with pyrophosphoric acid[15] (20 g.) was conducted at 80°C in a manner similar to that mentioned for 3-pyridylmethyl phosphate. After the addition of ethanol and ether to the reaction mixture, the oily material was separated from the clear supernatant liquid, dissolved in 15 ml. of water, and heated at 80°C for 45 min. Concentrated aqueous ammonia enough to convert the acid to the corresponding ammonium salt was added, and the salt was precipitated as white crystals by adding 200 ml. each of acetone and ether. The ammonium salt dissolved in a minimal volume of water was run onto a column of Dowex 50W-X8 [H^+] resin and eluted with water. The eluting behavior was found to be similar to that in 2-pyridylmethyl phosphate, as is shown in Fig. 2, and the product was recovered in a similar manner; recrystallization from ethanol-water (4 : 1); 3.8 g. of white rhombic prisms; extremely soluble in water and insoluble in ethanol; neutralization equivalence 94.6 (calcd. 94.6); λ_{max} 254 mμ (10^{-4} M aqueous solution), ε 4500.

Found: C, 38.06; H, 4.40; N, 7.28; P,* 15.2. Calcd. for $C_6H_8NO_4P$: C, 38.11; H, 4.26; N, 7.41; P, 16.4%.

The Separation of Phosphate Esters from an Artificial Mixture.—An aqueous solution containing 20 mg. each of the three isomeric esters in 4 ml. was applied to the top of a column (1.5×15 cm.) of Dowex 50W-X8 resin in the acid type (Fig. 1-A) and in the ammonium type (Fig. 1-B). The elution was conducted with water at the rate of 10 g. per hour. The effluents were classified according to their behavior in ultraviolet absorption, and each assembled portion was concentrated to dryness in vacuo. The measurements of the infrared and ultraviolet spectra as well as of the melting point were performed in order to identify such recovered material. With the ammonium-type resin, no 2-pyridylmethyl phosphate came off from the column, at least not while 400 g. of the effluent was being collected.

* One-quarter of the precipitate was purified by repeated precipitation with ethanol. The precipitate was dissolved in a small volume of water and reprecipitated with hot ethanol, the initial crop being removed. On repetition, the precipitate became somewhat crystalline; it was then suspended in boiling ethanol for an hour to remove the last traces of inorganic phosphoric acid, and finally recrystallized from ethanol-water.

* A sample for analysis was decomposed by heating it with concentrated nitric acid in a test tube. The amount of orthophosphoric acid thus formed was determined colorimetrically by the method of Allen (*Biochem. J.* (*London*), **34**, 858 (1940)). This method was applied to all the phosphates prepared in this work.

The Determination of Acid Dissociation Constants.—The acid dissociation constants of the three phosphate esters synthesized in this study were determined by potentiometric measurements. The apparatus and procedures have been described elsewhere.[16] The measurements were carried out at 25.0 ± 0.1°C and at the ionic strength of 0.10 M in potassium nitrate for two different concentrations of the esters, i. e., 1.00×10^{-3} M and 2.00×10^{-3} M, by ensuring that no hydrolysis occurred during the measurements. In accordance with the tribasic nature of the esters, three acid dissociation constants were defined in the usual way:

$$K_{H_3A}=\frac{[H_2A][H^+]}{[H_3A^+]};\quad K_{H_3A}=\frac{[HA^-][H^+]}{[H_2A]};\quad K_{HA}=\frac{[A^{2-}][H^+]}{[HA^-]}$$

where H_2A stands for the neutral ester species, and the brackets indicate the concentration. For the first approximation, K_{H_2A} and K_{HA} were calculated from the data obtained in a moderate pH range, where K_{H_3A} did not play any important role, in accordance with a usual algebraic method described previously.[17] These calculated values then permitted to evaluate the K_{H_3A} value from the data in the more acidic region. The three kinds of acid dissociation constants obtained in the above manner were then re-investigated to give the more satisfactory values listed in Table I.

16) Y. Murakami, This Bulletin, **35**, 52 (1962).
17) Y. Murakami, K. Nakamura and M. Tokunaga, ibid., **36**, 669 (1963).