boiled for 4.5 hours. Complete solution occurred after an hour. The clear solution after concentration *in vacuo* and evaporation with benzene yielded a resin. On long standing in dilute acetone this finally crystallized as rather soluble, delicate needles, m.p. 145–147° after preliminary sintering at 135°. For analysis it was dried at 110° and 0.2 mm.

Anal. Calcd. for $C_{33}H_{41}NO_9$: C, 66.54; H, 6.94. Found: C, 66.25; H, 7.06.

All analytical data have been obtained by Mr. D. Rigakos of this Laboratory.

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[CONTRIBUTION FROM NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Preparation of Crystalline Phosphorylated Derivatives of Vitamin B_6

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With a view to their use in projected enzyme experiments, the following derivatives of vitamin B_6 have been prepared in crystalline form: pyridoxamine-5-phosphate, pyridoxal-5-phosphate, pyridoxine-5-phosphate and deoxypyridoxine-5-phosphate. Phosphorylation was accomplished by heating pyridoxamine or deoxypyridoxine with a mixture of phosphoric acid and phosphorus pentoxide. Pyridoxal phosphate was prepared by the oxidation of pyridoxamine phosphate with finely divided manganese dioxide at room temperature, and pyridoxine phosphate was obtained from the same compound by deamination with nitrous acid. Purification was achieved in each case by chromatography on a weak cation-exchange resin and verified by paper chromatography in several solvent systems. The effectiveness of a similar column procedure in separating the phosphates of pyridoxamine, pyridoxal and pyridoxine from each other and from their unphosphorylated counterparts has been demonstrated. The ultraviolet absorption spectra of these compounds are essentially identical, with the exception of that of pyridoxal phosphate, which is distinguished by having a maximum at 385–390 mμ in neutral or alkaline solution, as has been reported by other investigators. These esters are relatively resistant to hydrolysis; little or no inorganic phosphate was split off on storage in aqueous solution in a refrigerator or freezer for 54 days. Data are presented to show their stability in acid and in alkali at 25 and 100°. The pure compounds were obtained in good yields, ranging from 40% for pyridoxal phosphate to 85% for deoxypyridoxine phosphate.

It is generally accepted that the biological activity of vitamin B_6 is due, in great part, to the participation of a phosphorylated derivative, pyridoxal-5-phosphate[1,2] as the coenzyme in many enzymatic reactions involving amino acids, namely, transamination,[3,4] decarboxylation,[5] desmolysis,[6] racemization,[7] etc. Although these systems have received considerable attention, the cofactors involved have, until recently, only been available as impure preparations. With the recognition that a clear understanding of the mechanisms by which the cofactors operate in the various reactions cited can best be reached by the employment of such cofactors in states of high purity, we have developed procedures leading to their preparation and to those of related compounds in pure crystalline condition.

The present paper describes the preparation and some properties of the free crystalline inner salts of pyridoxal-5-phosphate (PLP), pyridoxamine-5-phosphate (PMP), pyridoxine-5-phosphate (PNP) and deoxypyridoxine-5-phosphate (DPP). The very high activity of the first two compounds, PMP and PLP, as coenzymes and of the last two PNP and DPP, as enzyme inhibitors will be described in detail in a subsequent report.[8] The preparation of crystalline PMP and its ability to activate purified pig heart apotransaminase to the same extent as crystalline PLP have been noted in preliminary reports.[9-11]

Previous Methods of Preparation.—Pyridoxamine phosphate has been prepared by heating solutions of pyridoxal phosphate with glutamic acid[12] and by treating pyridoxamine in aqueous solution with phosphorus oxychloride.[13] Viscontini, Ebnother and Karrer have reported the preparation of the crystalline hydrochloride of pyridoxamine-5-phosphate by reaction of pyridoxamine dihydrochloride with metaphosphoric acid followed by mild hydrolysis.[14]

Gunsalus and co-workers have obtained pyridoxal phosphate as the crude barium salt after treatment of pyridoxal with phosphorus oxychloride.[15] Wilson and Harris[16] have phosphorylated pyridoxamine dihydrochloride with a phosphoric acid–phosphorus pentoxide mixture at room temperature. The pyridoxamine phosphate was not isolated but was converted to the impure ammonium salt of pyridoxal phosphate by oxidation with manganese dioxide, followed by charcoal adsorption and elution with ammonia. The barium and calcium salts of pyridoxal phosphate as well as the crystalline acridine salt were prepared[17,18] by the phos-

(1) W. W. Umbreit and I. C. Gunsalus, *J. Biol. Chem.*, **179**, 279 (1949).

(2) D. Heyl, E. Luz, S. A. Harris and K. Folkers, THIS JOURNAL, **73**, 3430 (1951).

(3) H. C. Lichstein, I. C. Gunsalus and W. W. Umbreit, *J. Biol. Chem.*, **161**, 311 (1945).

(4) D. E. O'Kane and I. C. Gunsalus, *ibid.*, **170**, 425 (1947).

(5) W. W. Umbreit, W. D. Bellamy and I. C. Gunsalus, *Arch. Biochem.*, **7**, 185 (1945).

(6) W. A. Wood, I. C. Gunsalus and W. W. Umbreit, *J. Biol. Chem.*, **170**, 313 (1947).

(7) W. A. Wood and I. C. Gunsalus, *ibid.*, **190**, 403 (1951).

(8) A. Meister, H. A. Sober and E. A. Peterson, *ibid.*, **206**, 89 (1954).

(9) E. A. Peterson, H. A. Sober and A. Meister, THIS JOURNAL, **74**, 570 (1952).

(10) A. Meister, H. A. Sober and E. A. Peterson, *ibid.*, **74**, 2385 (1952).

(11) E. A. Peterson, H. A. Sober and A. Meister, *Federation Proc.*, **11**, 268 (1952).

(12) E. E. Snell, THIS JOURNAL, **67**, 194 (1945).

(13) D. Heyl, D. Luz, S. A. Harris and K. Folkers, *ibid.*, **73**, 3436 (1951).

(14) M. Viscontini, C. Ebnother and P. Karrer, *Helv. Chim. Acta*, **34**, 2199 (1951).

(15) I. C. Gunsalus, W. W. Umbreit, W. D. Bellamy and C. E. Foust, *J. Biol. Chem.*, **161**, 743 (1945).

(16) A. N. Wilson and S. A. Harris, THIS JOURNAL, **73**, 4693 (1951).

(17) M. Viscontini, C. Ebnother and P. Karrer, *Helv. Chim. Acta*, **34**, 1834 (1951); **34**, 2199 (1951).

(18) M. Viscontini and P. Karrer, *ibid.*, **35**, 1924 (1952).

phorylation of the N,N-dimethylglycylhydrazone of pyridoxal with metaphosphoric acid followed by acid hydrolysis of the hydrazone. An unambiguous synthesis resulting in low yields of pyridoxal-5-phosphate was reported by Baddiley and Mathias,[19] involving the phosphorylation of the isopropylidene derivative of pyridoxine with a phosphoric acid–phosphorus pentoxide mixture, hydrolysis to pyridoxine phosphate and, finally, oxidation with manganese dioxide at 60°.

Crude pyridoxine phosphate has been prepared in poor yield as the calcium salt by the phosphorylation of pyridoxine in aqueous solution with phosphorus oxychloride.[4] Baddiley and Mathias[19] obtained pyridoxine-5-phosphate in solution during the course of their synthesis of pyridoxal-5-phosphate.

Deoxypyridoxine-5-phosphate was obtained in solution in poor yields by Beiler and Martin[20] and as the crude calcium salt by Umbreit and Waddell[21] after phosphorylation of deoxypyridoxine with phosphorus oxychloride.

Preliminary experiments in the present investigation had shown that phosphoric acid–phosphorus pentoxide mixtures such as those used by Plimmer and Burch[22] in the phosphorylation of aminoethanol, hydroxyproline, tyrosine and threonine were capable of phosphorylating the 5-position of pyridoxamine in good yields. When sufficient phosphorus pentoxide was added to react with the water in the 85% phosphoric acid employed (2.5:1 mixture of 85% H_3PO_4:P_2O_5) a temperature of 100° was required for adequate reaction (40% yield) in 24 hours.[9] The use of a 2:1 mixture of 85% phosphoric acid and phosphorus pentoxide at 80° for 15–20 hours resulted in a yield of 60%. High proportions of phosphorus pentoxide (P_2O_5): 85% H_3PO_4, 1:1.3) although resulting in increased amounts of polyphosphorylated material and therefore necessitating a preliminary partial hydrolysis with 1 *N* HCl, resulted in the most favorable yields (70–75%) even after heating for only two hours at 60°.

Initial efforts at oxidation of pyridoxamine-5-phosphate under the conditions described by Wilson and Harris,[16] *i.e.*, heating at 60° at *p*H 6.0 in an aqueous suspension of manganese dioxide, required about 50 minutes for complete disappearance of a ninhydrin spot test. A mixture of products was obtained and chromatographic separation on an ion-exchange column showed the presence of a major impurity poorly separated from the pyridoxal phosphate. The preparation of a more active form of manganese dioxide by the oxidation of manganous ion with potassium permanganate in the presence of celite, upon which the finely divided manganese dioxide was deposited, made possible a more rapid oxidation of pyridoxamine phosphate at room temperature and this impurity became a minor one. Although pure pyridoxamine phosphate (10 mg./ml.) could be oxidized completely in 30 minutes at 25° (or in 60 minutes at a concentration of 1 mg./ml.) by an equimolecular amount of manganese dioxide prepared in this way, by-products were less troublesome when a 3–5-fold excess of the oxidizing agent was used and the ester concentration was 2–3 mg./ml.

All four of the compounds described in this paper were purified by chromatography on a weak cation-exchange resin, employing water as the eluting agent.

Experimental

Pyridoxamine-5-phosphate (2-Methyl-3-hydroxy-4-aminomethyl-5-pyridylmethylphosphoric Acid).—Two grams of pyridoxamine dihydrochloride[23] (recrystallized from hot methanol to remove colored impurities) was mixed with 10 ml. of a solution of one part of phosphorus pentoxide in 1.3 parts of 85% phosphoric acid. The mixture was heated at 60° for two hours in a 250-ml. centrifuge bottle which was stoppered as soon as the initial evolution of HCl had subsided. After the addition of 1 ml. of water to the cooled reaction mixture, 50 ml. of absolute ethanol was slowly added with stirring, followed by 150 ml. of ether. The precipitate, consisting of a mixture of mono- and polyphosphorylated pyridoxamine with a small amount of the phosphate salt of the amine, was dissolved in 100 ml. of *N* HCl in a round bottomed flask and immersed in a rapidly boiling water bath for 20 minutes. Following evaporation *in vacuo*[24] to a volume of 5–7 ml., the hydrolysate was brought to *p*H 5–6 by the addition of concentrated ammonia and applied to the top of a 50–60 × 2.5 cm. column of Amberlite XE-64[25] in the washed acid form.[26]

Elution with water at the rate of 15–20 ml./hr. resulted in a separation of the components which was followed by measuring the optical density of the individual effluent fractions (10 ml. each) at 326 mμ (Fig. 1), as well as by ninhydrin treatment of samples from each tube dried on paper.[27] Water eluted two ninhydrin-reacting components. The second of these was by far the larger (see Fig. 1) and proved to be the desired pyridoxamine phosphate.

The tubes of this fraction[28] were combined and evaporated *in vacuo* to a volume of about 3 ml. After a few hours in the refrigerator, the mother liquor was separated from the crystalline product and the white crystals were washed with 3 ml. of ice-water, followed by absolute ethanol and then by ether. The crystals were equilibrated with room air for 24 hours before analysis. Less than 0.05% inorganic phosphorus was present.

Anal. Calcd. for $C_8H_{13}N_2O_5P\cdot 2H_2O$: C, 33.8; H, 6.0; N, 9.9; P, 10.9. Found: C, 34.2; H, 6.33; N, 10.0; P, 11.1.

The anhydrous product obtained by drying the dihydrate *in vacuo* over phosphorus pentoxide was very hygroscopic and unsuitable for weighing.

(19) J. Baddiley and A. P. Mathias, *J. Chem. Soc.*, 2538 (1952).

(20) J. M. Beiler and G. J. Martin, *J. Biol. Chem.*, **169**, 349 (1947).

(21) W. W. Umbreit and J. G. Waddell, *Proc. Soc. Exp. Biol. Med.*, **70**, 293 (1949).

(22) R. H. A. Plimmer and W. J. N. Burch, *Biochem. J.*, **31**, 398 (1937); **35**, 461 (1941).

(23) The commercial (Nutritional Biochemical Corporation, Cleveland, Ohio) hydrochlorides of pyridoxamine, pyridoxine and pyridoxal were recrystallized before use.

(24) All concentration procedures were performed *in vacuo* without permitting the internal temperature to rise above that of the room.

(25) Amberlite XE-64, an ion-exchange resin with carboxylic acid functional groups obtained from the Resinous Products Division, Rohm and Haas Company, Philadelphia 5, Penna.

(26) The resin was washed in a cycle of strong acid, water, strong alkali, water, then acid again. Since it was important to remove very finely divided material in order to obtain columns with low resistance to flow, repeated suspension and decantation were employed. After the washed resin was reacidified, it was poured into a glass column and rinsed with water until the effluent was no longer acid. A portion of the resin was removed at the alkaline stage and washed thoroughly with water for use in the pyridoxal phosphate preparation.

(27) Pyridoxamine and pyridoxamine phosphate form an orange color[9] with 0.25% ninhydrin in acetone (G. Toennies and J. J. Kolb, *Anal. Chem.*, **23**, 823 (1951)) after 10–20 minutes in a stream of warm air.

(28) Selection of the tubes of this fraction may be made entirely on the basis of the ninhydrin spot test. The two ninhydrin peaks indicated by bright red-orange spots, are separated by a long series of tan spots of much lower intensity. At the beginning of the second peak (pyridoxamine phosphate) a sharp increase in the intensity of the orange color above the low intensity of the intermediate spots occurs.

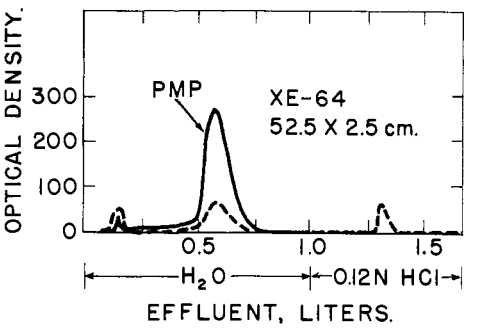

Fig. 1.—Purification of pyridoxamine-5-phosphate (PMP): solid line, 325 mμ; broken line, 295 mμ. Flow rate was 20 ml./hr. and 9–10 ml. fractions were collected.

From the mother liquor (generally a pale yellow) and the water wash above, an additional amount of slightly yellow crystalline product was obtained by the addition of alcohol and ether. Total yields of 70–75% were obtained in several trials.

If during recrystallization of pyridoxamine phosphate from hot water, crystals did not begin to form until the solution had nearly cooled to room temperature, short thick prisms of the dihydrate were obtained. However, when the compound was recrystallized from hot concentrated aqueous solutions, so concentrated that crystallization began when the temperature was still about 80°, fine needles were formed which gave the correct analysis for the anhydrous inner salt. The needles were equilibrated with room air for 24 hours before analysis. Less than 0.05% inorganic phosphorus was present.

Anal. Calcd. for $C_8H_{13}N_2O_5P$: C, 38.7; H, 5.24; N, 11.3; P, 12.5. Found: C, 38.9; H, 5.60; N, 11.2; P, 12.5.

Samples of these preparations when examined by paper chromatography as described below were homogeneous. They did not contain pyridoxal phosphate as determined with tyrosine apodecarboxylase.[5]

When a 60 × 2.5 cm. column was used, the more highly phosphorylated fraction emerged in the 100–250 ml. portion of the effluent, which also included a substance giving no color when treated with ninhydrin (Fig. 1). Pyridoxamine phosphate was found mainly in the 500–1,000 ml. range. Pyridoxamine and basic impurities remained on the column and required elution with 0.1 *N* hydrochloric acid. Thus, separation was sufficiently great to permit collection of the water effluent in two portions, a matter of some importance when a fraction collector is not available. In experiments employing this procedure, the first fraction (about 300 ml.) included a colorless acidic effluent, followed by a pale yellow non-acidic one. The second fraction (usually 500–600 ml.) was collected until the effluent no longer gave a positive ninhydrin[27] spot test for pyridoxamine phosphate. Evaporation and crystallization were carried out as described above. Pyridoxamine phosphate obtained by this procedure should be recrystallized from hot water as very small amounts of readily soluble impurities are included in this fraction. The yields obtained are comparable with those resulting from the use of the more careful fractionation.

Pyridoxal-5-phosphate (2-Methyl-3-hydroxy-4-formyl-5-pyridylmethylphosphoric Acid).—Crude pyridoxamine phosphate was prepared from 2 g. of recrystallized pyridoxamine dihydrochloride as described above, carrying the procedure as far as the evaporation *in vacuo* of the partially hydrolyzed product. The resulting sirup was diluted with 50 ml. of water and adjusted to about *p*H 5 with 1 *N* NaOH, then made up to 200 ml. with water. In experiments employing pure pyridoxamine phosphate, 1.7–2.0 g. of the dihydrate was dissolved in 200 ml. of water and used without *p*H adjustment.

The oxidizing agent was prepared by adding a solution of 1.80 g. of potassium permanganate (11.4 mmoles) in 150 ml. of water in a continuous thin stream to a constantly agitated suspension of 20 g. of celite[29] in 150 ml. of a solution containing 3.50 g. of manganous chloride ($MnCl_2 \cdot 4H_2O$, 17.7 mmoles). During the addition, which took less than ten minutes, finely divided manganese dioxide was deposited upon the celite to form a brown suspension. After standing about half an hour, the mixture was filtered with suction without washing the filter cake and without allowing it to become dry.

The MnO_2–celite was resuspended in sufficient water to make 300 ml. and was then rapidly mixed with the pyridoxamine phosphate solution in a beaker. The mixture was stirred continuously at 25° and samples removed at intervals were spotted on filter paper, the first being taken 15 minutes after mixing the reactants, the others at 5-minute intervals thereafter. Each spot was immediately treated with 0.25% ninhydrin in acetone and hung before a warm-air fan for development of color. The reaction was considered to be complete when a spot test made 5 minutes previously showed only the yellow color of pyridoxal phosphate with no indication of orange.

Sufficient Amberlite XE-64 in the sodium form[26] was then added to bring the *p*H to 7.0–7.4[30] and the mixture was filtered with suction. The filter cake was thoroughly washed on the filter with water and the amber filtrate and washings were concentrated *in vacuo*.[24] The dark concentrate (25 ml.)[31] was then carefully applied to a 100 × 3.7 cm. Amberlite XE-64 (acid form) column and eluted with water at the rate of 50 ml./hr. The effluent was collected in 10–20 ml. fractions and a representative elution curve is presented in Fig. 2A. In some cases the amount of impurity was somewhat larger. Each of the fractions was examined spectrophotometrically at 275, 295, 325 and 385 mμ, and an aliquot (about 0.01 ml.) of each of the colored fractions was dried on heavy filter paper. Pyridoxal phosphate, readily distinguished from the other colored products by its bright yellow color on the paper, appeared in the fractions bracketed by the two arrows in Fig. 2A. This color

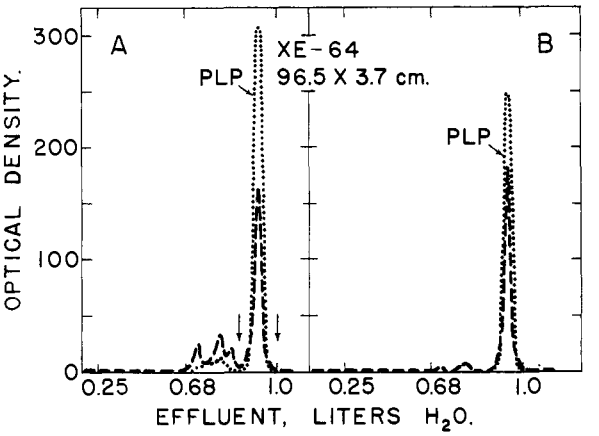

Fig. 2.—Purification of pyridoxal-5-phosphate (PLP): dotted line, 385 mμ; broken line, 295 mμ. Flow rate was 50 ml./hr. and 10-ml. fractions were collected. The difference in magnitude of the peaks in A and B should not be taken as a measure of loss in rechromatography since the extinction coefficient is markedly dependent upon concentration in unbuffered solutions.

was intensified by exposure to ammonia, which also caused the spots to fluoresce with a bright yellow light when examined under an ultraviolet lamp.[32] All of the fractions containing pyridoxal phosphate (including those on both sides contaminated with small amounts of other material, *i.e.*, between the arrows in Fig. 2A) were combined for rechro-

(29) Celite Analytical Filter-Aid, Johns Manville.

(30) This converted the pyridoxal phosphate to the very soluble sodium salt and largely freed the solution of manganous ion formed during the reaction. Further oxidation and polymerization were, moreover, minimized by neutralization.

(31) Concentration below this volume may cause precipitation of some of the large amount of inorganic phosphate present at this stage when crude pyridoxamine phosphate is oxidized. When the pure ester is used, this volume should be 10 ml. or less.

(32) Hanovia Inspectolite lamp (Hanovia Chemical and Manufacturing Company, Newark 5, N. J.) No. SC-5041 with a filtered (to pass 3660 Å.) EH-4 arc tube.

matography[33] and adjusted to *p*H 7 with 1 *N* sodium hydroxide.

The solution was evaporated *in vacuo* to small volume just before application in about 10 ml. to the same resin column used before, but which had in the meantime been washed with 2 *N* hydrochloric acid and rinsed with water until free of acid. Elution with water was carried out as before and the fractions were examined as above. Spectrophotometric as well as spot test examination revealed that the pyridoxal phosphate was well separated from neighboring impurities (Fig. 2B). The spot test alone may be sufficient for the first column chromatography but spectrophotometric observation should be employed for the second.

The selected fractions were combined and concentrated *in vacuo*, then transferred (with rinses to make a total volume of 10–15 ml.) to a cellophane sac and dialyzed with internal agitation against one liter of water for about 15 hours in the cold. The dialyzate was evaporated *in vacuo* to 80–100 ml. and then lyophilized.[34] A lemon yellow mat of microscopic needles of pyridoxal phosphate monohydrate was obtained in 40% of the theoretical yield calculated from pyridoxamine dihydrochloride. These needles were equilibrated with room air for 24 hours before analysis; 0.13% inorganic phosphorus was found.

Anal. Calcd. for $C_8H_{10}O_6NP \cdot H_2O$: C, 36.2; H, 4.52; N, 5.28; P, 11.7. Found: C, 36.3; H, 4.45; N, 5.41; P, 11.9.

Crystallization of pyridoxal-5-phosphate could be induced from concentrated aqueous solutions by the addition of alcohol and ether, but only small crystals were obtained and the yields were low, hence lyophilization was used to obtain the final product. Yellow needles of the sodium salt of pyridoxal-5-phosphate could be obtained by the slow addition of absolute alcohol to a concentrated solution of the ester brought to *p*H 7.5 by the addition of sodium hydroxide before evaporation. On standing, these grew into readily visible clusters.

Paper chromatographic examination as described below, showed the presence of trace amounts of pyridoxal and a substance remaining at the origin in all solvents which was probably polymeric material.

Pyridoxine-5-phosphate (2-Methyl-3-hydroxy-4-hydroxymethyl-5-pyridylmethylphosphoric Acid).—Pyridoxamine phosphate dihydrate (1.0 g.) was dissolved in 100 ml. of 0.1 *N* hydrochloric acid.[35] Sodium nitrite (0.5 g.) in 100 ml. of water was added and the mixture was allowed to stand at 25° for 6 hours. A sample taken at this time and dried on filter paper failed to develop an orange color, when treated with ninhydrin, indicating the essentially complete disappearance of pyridoxamine phosphate. To destroy excess nitrous acid, 200 mg. of urea was added and the solution was allowed to stand overnight or until a negative test was obtained with starch–iodide paper. It was then evaporated to small volume *in vacuo*, adjusted to *p*H 5–6 with 10 *N* sodium hydroxide, and applied in a volume of about 10 ml. to a 100 × 3.7 cm. Amberlite XE-64 (acid form) column. Effluent fractions obtained on elution with water at the rate of 50 ml./hr. were examined spectrophotometrically at 295 and 326 mμ and the pyridoxine phosphate was found to be well separated from a pale yellow forepeak (Fig. 3). The positions of the eluted materials could also be determined by the fluorescence of samples dried on filter paper when examined under ultraviolet light[32] during exposure to ammonia vapors.

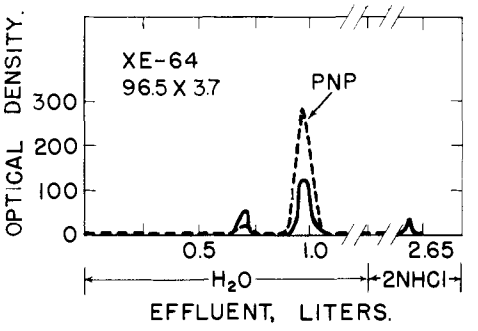

Fig. 3.—Purification of pyridoxine-5-phosphate (PNP): solid line, 325 mμ; broken line, 295 mμ. Flow rate was 50 ml./hr. and 10-ml. fractions were collected.

Fractions containing the major 295 mμ absorbing component (the second peak, Fig. 3) were combined and evaporated *in vacuo* to about 3–4 ml. White needles crystallized. The flask was allowed to stand in the refrigerator a few hours, then alcohol was added to complete the precipitation. The crystals were washed with alcohol, then with ether, and equilibrated with room air for 24 hours before analysis. The yield was 70% of theory and no inorganic phosphorus was found. Paper chromatographic examination as described below indicated homogeneity.

Anal. Calcd. for $C_8H_{12}O_6NP$: C, 38.5; H, 4.86; N, 5.62; P, 12.4. Found: C, 38.5; H, 4.84; N, 5.81; P, 12.75.

Deoxypyridoxine-5-phosphate (2,4-Dimethyl-3-hydroxy-5-pyridylmethylphosphoric Acid).—A mixture of 1.5 g. of deoxypyridoxine hydrochloride[36] and 10 ml. of one part phosphorus pentoxide in 1.3 parts of 85% phosphoric acid was heated at 60° for two hours. The viscous product was dissolved in 100 ml. of 1 *N* hydrochloric acid and immersed in a rapidly boiling water-bath for 30 minutes, then cooled and diluted to 250 ml. with water. About 30 g. of Darco G-60[37] was added and the suspension was stirred for a few minutes. When the optical density of a test filtrate was found to be less than 0.700 at 280 mμ, the entire suspension was filtered slowly through Whatman no. 50 paper with suction. The carbon filter cake was washed with water (about 700 ml.) until the effluent was just faintly acid, and the filtrate and washings were discarded. The cake was then extracted on the filter with 2% ammonia (about 1 liter, at the rate of 1–2 drops per second) until the optical density of the emerging liquid was less than 0.400 at 300 mμ. The filter cake was not permitted to run dry throughout this procedure.

The ammonia eluate was evaporated *in vacuo* to 10–15 ml. Solid material which appeared on the walls of the flask redissolved upon warming. This solution was transferred to the top of a 98 × 3.7 cm. column of Amberlite XE-64 (acid form) and eluted with water at the rate of 50 ml./hour. Examination of the effluent fractions showed two widely separated substances absorbing at 280 mμ (Fig. 4). The

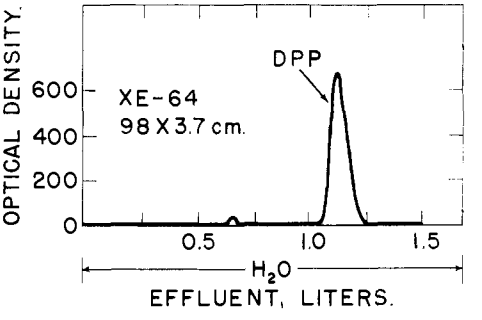

Fig. 4.—Purification of deoxypyridoxine-5-phosphate (DPP): solid line, 280 mμ. Flow rate was 60 ml./hr. and 10-ml. fractions were collected.

(33) Preparations employing pure pyridoxamine-5-phosphate as the starting material yielded pyridoxal-5-phosphate sufficiently pure for most purposes although traces of impurities could still be removed by rechromatography. When crude pyridoxamine phosphate containing large amounts of inorganic phosphate was used (*i.e.*, immediately after the preliminary partial hydrolysis) rechromatography of the pyridoxal phosphate fraction was necessary. However a final product of highest purity could be obtained in this manner, indistinguishable from that obtained from pure starting material and in slightly higher over-all yield.

(34) While dialysis before lyophilization removed most of the polymeric material, there is little doubt that small amounts of the dialyzable lower polymers of pyridoxal phosphate are still present. Complete elimination of this type of impurity may well be impossible.

(35) It was necessary to use a dilute solution of pyridoxamine phosphate in order to minimize the formation of an unidentified side product (possibly arising from a coupling reaction) which, however, was easily separated from the pyridoxine phosphate on the ion-exchange column.

(36) Kindly donated by Merck and Co., Rahway, N. J.

(37) Since alcohol precipitation of the organic material was not feasible, a charcoal adsorption procedure based on that used by Wilson and Harris[16] was employed.

first peak (650–700 ml. portion of the effluent) was present in very small amount, and crystals of the second substance, deoxypyridoxine phosphate, were obtained in 85% yield upon evaporation of the 1050–1330 ml. portion to small volume, and addition of alcohol and ether to complete precipitation. The crystals were washed with ether and equilibrated with room air for 24 hours before analysis. Paper chromatographic examination as described below indicated homogeneity. No inorganic phosphorus or ammonia nitrogen was found.

Anal. Calcd. for $C_8H_{12}O_5NP \cdot H_2O$: C, 38.2; H, 5.62; N, 5.58; P, 12.3. Found; C, 38.5; H, 5.82; N, 5.40; P, 12.6.

Results and Discussion

Separation of an Artificial Mixture.—In order to establish the effectiveness of the chromatographic procedure in separating any of the vitamin B_6 compounds from the others, a solution of pyridoxal phosphate, pyridoxamine phosphate, pyridoxine phosphate, their unphosphorylated counterparts[23] and pyridoxic acid,[38] was chromatographed on an XE-64 (acid) column in the manner represented in Fig. 5. The identity of each peak was determined by examination of the effluent fractions at four wave lengths (260, 295, 325 and 385 mμ) in both acidic and basic solutions, by the color reactions described below, and by individual absorption spectra of several fractions within each peak. It can be seen from the figure that the phosphate esters were individually resolved and emerged much earlier than the corresponding unphosphorylated compounds, and that although pyridoxic acid appeared between the pyridoxine and the pyridoxamine phosphate peaks, it too was clearly separated.

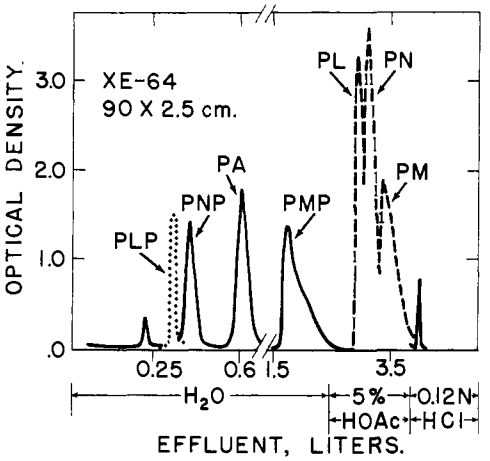

Fig. 5.—Separation of an artificial mixture: 125 mg. of a mixture of PMP, PLP, PNP, DPP, pyridoxamine (PM), pyridoxal (PL), pyridoxine (PN) and pyridoxic acid (PA) were added to the column and eluted at a flow rate of 6 ml./hr. and collected in 4-ml. fractions. Only selected wave lengths are plotted. Solid line, 325 mμ; broken line, 295 mμ; dotted line, 385 mμ.

The preparation of pyridoxamine phosphate labeled with P^{32} provided a further test of the homogeneity of this compound. A plot of the radioactivity of the effluent fractions comprising the major peak followed closely the curve representing the optical density of these fractions at 325 mμ (Fig. 6).

(38) Pyridoxic acid (2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine) was prepared from the oxime of pyridoxal (D. Heyl, THIS JOURNAL, **70**, 3434 (1948)).

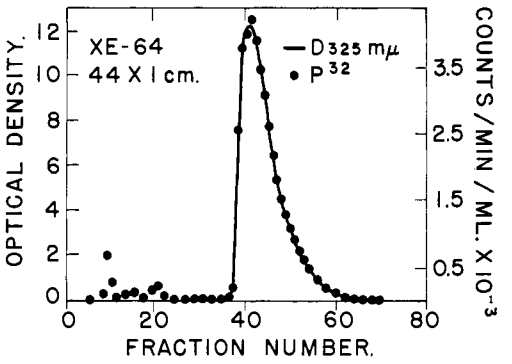

Fig. 6.—Radioactivity of effluent fractions of P^{32}-labeled pyridoxamine-5-phosphate. After preliminary purification on a shorter column to remove inorganic phosphate, the material was added to the column and eluted with water at a flow of 6 ml./hr. and collected in 3-ml. fractions.

Ultraviolet Absorption Studies.—The ultraviolet absorption spectra of the four phosphorylated vitamin B_6 derivatives are given in Fig. 7. The pyridoxal phosphate spectra are in general agreement with those reported by other investigators[2,17,39] although quantitative differences exist. All show a shift in the position of the maxima to lower wave lengths on acidification. The spectrum of pyridoxal phosphate at *p*H 7.0 or in alkali differs from the others in having a maximum at 388 mμ which is apparently specific for the –CHO group in this series of compounds. The spectra of the other three esters are essentially identical with each other, except that the lack of the –OH group in the 4-position has shifted the 294 mμ maximum (0.1 *N* HCl) to 283 mμ in the case of deoxypyridoxine phosphate.

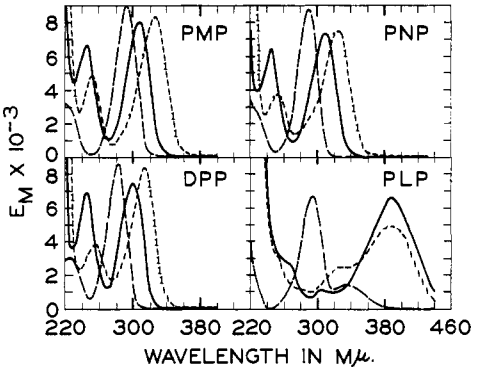

Fig. 7.—Effect of *p*H on absorption spectra. Spectra were determined in solutions containing 40–44 μg./ml. in 1-cm. silica cuvettes in a Beckman DU spectrophotometer. Solid line, 0.1 *N* NaOH; short dashed line 0.1 μ, *p*H 7.0 sodium phosphate buffer; broken line, 0.1 *N* HCl.

In Table I is presented a comparison of the molar extinction coefficients at maxima of the four phosphorylated compounds and their unphosphorylated counterparts. The generalizations stated above are also seen to hold in the absence of the phosphate group as well. With the exception of pyridoxal, the introduction of the phosphate group at the 5-position seems to effect no change in the spectra ex-

(39) W. W. Umbreit, D. J. O'Kane and I. C. Gunsalus, *J. Biol. Chem.*, **176**, 629 (1948).

cept for a small increase in the molar extinction coefficient. The esterification of the 5-position of pyridoxal with phosphoric acid reduced the molar extinction coefficient at the maximum in the lower wave length, but the magnitude of the maximum at the higher wave length was considerably augmented.

TABLE I

COMPARISON OF THE MOLAR EXTINCTION COEFFICIENTS OF PHOSPHORYLATED AND UNPHOSPHORYLATED VITAMIN B_6 COMPOUNDS

	Molar extinction coefficients[a]					
	0.1 *N* HCl		pH 7.0[c]		0.1 *N* NaOH	
Compound[b]	Max., mμ	E_M	Max., mμ	E_M	Max., mμ	E_M
Pyridoxamine phosphate dihydrate	293	9000	253	4700	245	6700
			325	8300	308	8000
Pyridoxamine dihydrochloride[d]	293	8500	253	4600	245	5900
			325	7700	308	7300
Pyridoxal phosphate monohydrate	295	6700	330	2500	305	1100
			388	4900	388	6600
Pyridoxal hydrochloride[d]	288	8600	318	8200	300	5800
			390	200	393	1700
Pyridoxine phosphate	290	8700	253	3700	245	6500
			325	7400	310	7300
Pyridoxine hydrochloride[d]	290	8400	253	3700	245	6500
			325	7100	308	7000
Deoxypyridoxine phosphate monohydrate	283	8600	253	3800	245	6900
			315	8400	300	7500
Deoxypyridoxine hydrochloride[d]	283	8200	255	3900	245	6900
			315	7800	300	7000

[a] Beckman DU spectrophotometer in 1-cm. silica cuvettes. [b] Concentration in the cuvette, 40–44 μg./ml. [c] 0.10 *M* sodium phosphate buffer. [d] Recrystallized commercial preparations.

Paper Chromatographic Studies.—Of the several solvents[40] and papers[41] tried, the formic acid mixture (Formix) was the most generally satisfactory solvent in that all four phosphorylated esters[42] were nicely separated on either W-1 or SS 598 paper (Table II). Pyridoxal phosphate and pyridoxamine phosphate as well as their unphosphorylated counterparts were well resolved in the lutidine mixture. However, pyridoxine phosphate and deoxypyridoxine phosphate, when present, overlapped with the pyridoxamine phosphate area. The other solvents were able to separate pyridoxal and pyridoxamine phosphates but were not able to prevent overlapping of the pyridoxal phosphate area by the remaining two esters.

In alkaline developing solvents, pyridoxal and pyridoxal phosphate are yellow and could be seen on the untreated paper chromatogram, the phosphorylated compound appearing considerably stronger. The color was intensified by exposure of the paper to ammonia, particularly when acidic developing solvents were used. Under ultraviolet light[32] pyridoxal, pyridoxamine, their phosphate esters and pyridoxine were easily seen as a bluish fluorescence. Exposure to ammonia vapor intensified the fluorescence of all these compounds (pyridoxal phosphate emitted a vivid yellow light) and permitted the visualization of pyridoxine phosphate. The augmentation of fluorescence was transient but could be restored by re-exposure to ammonia. Deoxypyridoxine and its 5-phosphate were never seen by this procedure.

The phosphorylated compounds could be detected on the paper chromatogram by any of the tests suggested for the detection of phosphate esters.[43–46] The Pauly reaction as modified by Sanger and Tuppy[47] is non-specific in that all four of these substances react to give tan colors.

TABLE II

RELATIVE MOVEMENT ($R_F \times 100$) ON PAPER CHROMATOGRAMS

	Formic acid mixture		Lutidine mixture	
	W-1	SS-598	W-1	SS-598
Pyridoxamine phosphate	20–30	24–36	2–8	0–6
Pyridoxal phosphate	33–42	42–52	16–22	26–31
Pyridoxine phosphate	47–52	56–62	...	3–11
Deoxypyridoxine phosphate	54–60	63–72	4–10	8–15
Pyridoxamine	58–69	40–58	58–65	60–66
		60–66[a]		
Pyridoxal	70–80	78–88	83–90	82–87
Pyridoxine	74–89	80–88	78–83	76–80
Deoxypyridoxine	74–80	84–91	82–90	77–85

[a] Chromatograms of commercial pyridoxamine dihydrochloride, when treated with ninhydrin, showed two orange spots of roughly equal intensity and a faster minor yellow component. Recrystallization removed the yellow contaminant. Chromatography of recrystallized pyridoxamine as the free base or as its salt with phosphoric acid resulted in only one spot. The dihydrochloride, however, made from the recrystallized free base, still produced two orange areas.

TABLE III

PER CENT. RELEASE OF INORGANIC PHOSPHATE[a,b]

	Phosphate ester of			
Conditions	Pyridoxamine	Pyridoxal	Pyridoxine	Deoxypyridoxine
Freezer, −17°, 54 days	<0.4	...	<0.4	<0.4
Refrigerator, 7°, 54 days	<0.4	2.1	2.6	<0.4
Incubator, 37°, 54 days	8.5	26.2	15.3	7.2
Room temp., 25°, 54 days	...	15.1	7.8	..
1 *N* HCl, 25°, 54 days	1.5	9.0	1.0	0.8
1 *N* NaOH, 25°, 54 days	3.1	2.3	2.6	5.2
1 *N* HCl, 100°, 15.3 hr.	23	73	35	26
0.05 *N* HCl, 100°, 15.3 hr.	42	82	30	26
1 *N* NaOH, 100°, 15.3 hr.	10	6	11	5
0.05 *N* NaOH, 100°, 15.3 hr.	7	5	9	4
6 *N* H_2SO_4, 100°	50% in 9 hr.	50% in 4 hr.	55% in 19 hr.	50% in 21 hr.

[a] Total and inorganic phosphorus determined by the methods of D. F. Boltz and M. G. Mellon, *Anal. Chem.*, 19, 873 (1947), and J. B. Martin and P. M. Doty, *ibid.*, **31**, 965 (1949), respectively. [b] Phosphate esters were at a concentration of 1.2–1.9 mg./ml.

(40) Lutidine mix, consisting of 2,6-lutidine (55 parts by volume) water (25 parts), ethanol (20 parts) and diethylamine (1 part); Formix, consisting of 70 parts *t*-butyl alcohol, 15 parts of water and 15 parts of 89% formic acid; and 77% ethanol (R. J. Block, *Anal. Chem.*, **22**, 1327 (1950)). The acidic and basic methanol solvents of R. S. Bandurski and B. Axelrod (*J. Biol. Chem.*, **193**, 405 (1951)) and the 1-propanol-ammonia mixture of C. S. Hanes and F. A. Isherwood (*Nature*, **164**, 1107 (1949)).

(41) Whatman No. 1 and No. 4; Schleicher and Schuell 598.

(42) All these compounds and their unphosphorylated counterparts were tested at 0.01 and 0.03 ml. of a 2 mg./ml. solution.

(43) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).

(44) C. S. Hanes and F. A. Isherwood, *Nature*, **164**, 1107 (1949).

(45) S. Burrows, F. S. M. Grylls and J. S. Harrison, *ibid.*, **170**, 800 (1952).

(46) H. E. Wade and D. M. Morgan, *ibid.*, **171**, 529 (1953).

(47) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).

Hydrolysis of the Compounds.—The release of inorganic phosphorus from the four phosphorylated compounds of the vitamin B_6 series under various conditions is briefly outlined in Table III. Although pyridoxal phosphate was the most labile of the four, only 2% of its total phosphorus was detected as inorganic phosphorus after 54 days storage of an aqueous solution in a refrigerator or a freezer. In agreement with observations based on biological growth data[48] pyridoxal phosphate was more acid labile than pyridoxamine phosphate. Storage at room temperature for 54 days in 1 *N* HCl resulted in only 9% hydrolysis of the phosphate linkage in pyridoxal phosphate and only about 1% for the other compounds. In 1 *N* NaOH on the other hand pyridoxal phosphate appeared to be more stable.

(48) J. C. Rabinowitz and E. E. Snell, *J. Biol. Chem.*, **169**, 643 (1947).

At 100°, the difference between alkaline and acid hydrolysis was most evident (see Table III). In acid, the phosphoric ester bond was least resistant to hydrolysis in pyridoxal phosphate and most resistant in deoxypyridoxine phosphate. In alkali, on the other hand, although the ester bond in general was much more resistant to hydrolysis, pyridoxal phosphate was one of the more stable compounds. Pyridoxal and pyridoxamine phosphates were more rapidly hydrolyzed in dilute than in concentrated acid; none showed increased lability in dilute alkali. The last line in the table presents an estimate of the half lives of these compounds in 6 *N* sulfuric acid at 100°. Complete hydrolysis was obtained under these conditions in about 30 and 55 hours for the pyridoxal and pyridoxamine phosphates, respectively.

We are grateful to R. J. Koegel and his staff for the analyses presented in this paper.

[CONTRIBUTION FROM DEPARTMENT OF CHEMISTRY, UNIVERSITY OF ILLINOIS]

The Stereochemistry of α-Haloketones. V. Prediction of the Stereochemistry of α-Brominated Ketosteroids

BY ELIAS J. COREY

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Methods are presented for predicting the orientation of bromine in all α-bromoketosteroids with ketone function in ring A, B or C and A/B *cis* or *trans*. One method applies to α-bromoketosteroids whose stereochemistry is thermodynamically controlled and the other method applies to α-bromoketosteroids whose stereochemistry is kinetically controlled. In every case there is agreement between predicted and determined configuration at $C_{(Br)}$. A number of cases are reported in which prediction has led to a redetermination and eventual reassignment of configuration. In other cases, configurations, which are consistent with predictions, are assigned for the first time using both infrared and chemical evidence. Thus it has been shown, in accord with expectation, that the bromination of 5α,6β-dibromocholestane-3-one produces the 4α-derivative faster than the 4β-derivative although the latter is the more stable. Likewise the bromination of 3α-acetoxycholestane-6-one affords, as predicted, the 5α-bromo derivative which is isomerized to the 7α-bromo derivative by hydrogen bromide. The assignment of α-orientation to the bromine in the latter substance, which conflicts with previous reports, has been proven by infrared absorption and transformation of the bromoketone to 3β-acetoxy-6β-hydroxy-7α-bromocholestane and thence to 3β-hydroxy-6,7β-oxidocholestane. The stereochemistry of the oxide was proved by reduction with lithium aluminum hydride to 3β,6β-dihydroxycholestane. The 5,7-dibromo derivatives of 3α-acetoxycholestane-6-one, m.p. 152° and m.p. 129°, have been shown to be, respectively, the 5α,7α- and 5α,7β-bromoketones. The product of bromination of methyl 3α,12α-diacetoxy-7-ketocholanate under conditions of thermodynamic control is the 6α-bromo epimer and not the β-epimer as previously reported.

Although the chemistry of α-bromoketosteroids has been the subject of much research, there have appeared no rules or methods for predicting whether the bromination of a given ketosteroid will lead to that epimer in which bromine is α-oriented, that in which bromine is β-oriented or a mixture of the two. Furthermore, as is now known, there are several instances in which the assignment of configuration to a bromoketosteroid, even after preparation and chemical study, has been incorrect. Another point of difficulty is the lack of distinction in the literature between thermodynamic (equilibrium) control of reaction product and kinetic (rate) control of reaction product. Such a distinction is important here because it may be possible for the primary bromination product (or products) to undergo thermodynamic equilibration by enolization. There are just a few cases in the literature where it is clear whether the bromoketone described is the stable or the unstable epimer. Obviously, any general method for predicting the stereochemistry of α-brominated ketosteroids must provide for both the thermodynamically and kinetically controlled products.

Recently, we have described in brief a method for deducing the more stable orientation of bromine in a bromoketosteroid.[1] This in turn gives the orientation which will occur in the thermodynamically controlled bromination product. In addition a rule has been proposed for predicting the stereochemistry of the kinetically controlled bromination product of a ketosteroid.[1] The present paper is concerned with the details of the application of these methods to specific α-bromoketosteroids.[2]

Thermodynamically Controlled Bromination Products.—It has been shown previously[3] that in the case of an α-bromocyclohexanone electrical repulsions involving carbon–oxygen and carbon–bromine dipoles destabilize the bromine-equatorial form relative to the bromine-polar form. On the

(1) E. J. Corey, *Experientia*, **9**, 329 (1953).

(2) The case of 2α-bromocholestane-3-one has already been discussed; E. J. Corey, THIS JOURNAL, **75**, 4832 (1953).

(3) E. J. Corey, *ibid.*, **75**, 2301 (1953).