45. *The Photolysis of Pyridoxal Phosphate.*

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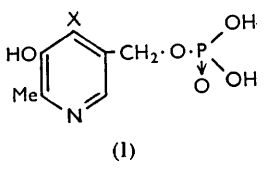
Pyridoxal phosphate, the co-factor of many enzymes involved in amino-acid metabolism, has been found to undergo a rapid, benzoin type, self-condensation in oxygen-free solution when exposed to light. The main product isolated was shown to be 5 : 5′-bis(dihydroxyphosphinyloxymethyl)-3 : 3′-dihydroxy- 2 : 2′-dimethyl-4 : 4′-pyridil, presumably formed from the corresponding pyridoin during working up in the presence of air.

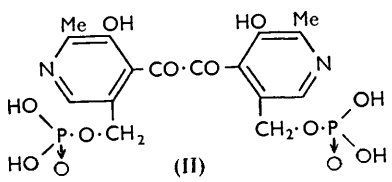
Aqueous solutions of pure crystalline pyridoxal phosphate monohydrate[1] (I; X = CHO) were found to decompose rapidly when exposed to sunlight or irradiated with ultraviolet light. Dilute solutions (*ca.* 0·5%) in air-free water under nitrogen, exposed to strong summer sunlight in 100 ml. Pyrex flasks, were completely photolysed in one hour. Little difference in the rate of reaction was observed between pH 2·0 and pH 8·0.

The photolysis was followed by measuring the ultraviolet absorption spectra of the solutions at pH 7·0 in phosphate buffer, the characteristic maximum of pyridoxal phosphate at 387 mμ being replaced by one of approximately equal intensity at 288 mμ. In acid or in alkaline solution the difference between the spectra of pyridoxal phosphate and its photolysed solution was much less marked.

By fractionation on a carboxylic acid ion-exchange resin, the principal product of the reaction was isolated in 75—88% yield, as colourless, high-melting crystals with an elementary analysis approximating to that of pyridoxal phosphate. It was almost insoluble in water and most organic solvents but dissolved readily in aqueous alkali to intensely yellow solutions.



(I)



(II)

The presence of one carbonyl group per atom of phosphorus in the molecule was established by preparing an oxime with a N : P ratio of 2 : 1. Whereas pyridoxal phosphate reacts rapidly with hydroxylamine at room temperature, the oxime of the photolysis product was formed only on several hours' boiling. The reactive 4-formyl group of pyridoxal phosphate had therefore been altered.

[1] Peterson and Sober, *J. Amer. Chem. Soc.*, 1954, **76**, 169; Long, B.P. 749,800.

Oxidation of pyridoxal phosphate with alkaline hydrogen peroxide gives 3 : 4-dihydroxy-2-methyl-5-pyridylmethyl dihydrogen phosphate [2] (I; X = OH). Oxidation of the photolysis product by this method gave a tribasic acid, identical with the compound made by oxidising pyridoxamine 5-(dihydrogen phosphate) (I; X = $CH_2 \cdot NH_2$) with excess of manganese dioxide at pH 2·0. This acid was shown to be 4-carboxy-3-hydroxy-2-methyl-5-pyridylmethyl dihydrogen phosphate (4-pyridoxic acid phosphate) (I; X = CO_2H), hydrolysis with concentrated hydrochloric acid giving the known lactone [3] of 4-carboxy-3-hydroxy-5-hydroxymethyl-2-methylpyridine. Since *o*-phenolic aldehydes on oxidation with alkaline hydrogen peroxide invariably give *o*-dihydroxy-compounds,[4] oxidation of the photolysed pyridoxal phosphate to a pyridine-4-carboxylic acid suggested that, while a carbonyl group was still in the 4-position in this compound, it was no longer part of an aldehyde. Oxidation with hydrogen peroxide in alkaline solution, followed by electrophoresis on filter paper, can be used to detect photolysis of pyridoxal phosphate since the phosphate of the carboxylic acid is separated readily from that of the dihydric phenol.

The photolysis product was reduced with sodium borohydride to a glycol, isolated in two fractions each associated with borate, and apparently differing only in the ease with which this was removed. Both forms gave pyridoxal phosphate on oxidation with periodate and were therefore presumably stereoisomers of 5 : 5′-bis(dihydroxyphosphinyloxymethyl)-3 : 3′-dihydroxy-2 : 2′-dimethyl-4 : 4′-hydropyridoin which has two asymmetric carbon atoms. In the oxidation, 1·2 moles of periodate per mole of glycol were used. Subsequently it was found that pyridoxal phosphate consumes 0·15 mole of periodate per mole under identical conditions. Comparison of the reaction mixtures by electrophoresis on filter paper showed them to have the same qualitative composition.

From these experiments it was concluded that the compound being investigated was 5 : 5′-bis(dihydroxyphosphinyloxymethyl)-3 : 3′-dihydroxy-2 : 2′-dimethyl-4 : 4′-pyridil (II). This was confirmed by oxidising the compound with sodium periodate to 4-pyridoxic acid 5-(dihydrogen phosphate) as the only product, the periodate uptake being 0·5 mole per atom of nitrogen. This reaction was extremely fast, in contrast to the oxidation of benzil which requires some days for completion.[5] Under the influence of light and in the absence of air pyridoxal phosphate therefore undergoes a benzoin condensation. The substitution in all four α-positions of the pyridoin initially formed would be expected to stabilise the ene-diol structure,[6] and the oxidation of ene-diol forms of α-pyridoins by atmospheric oxygen has been described by Eistert and Munder.[7] The isolation of a pyridil rather than a pyridoin is therefore not surprising.

The lack of colour in the solid pyridil is in keeping with previous observations that benzils with *o*-hydroxy-substituents can exist in stable, colourless modifications.[8]

When pyridoxal phosphate is decomposed by light in the presence of air, the pyridil (II) is always accompanied by some 4-pyridoxic acid 5-(dihydrogen phosphate). If a vigorous stream of oxygen is passed through the solution undergoing photolysis, the carboxylic acid becomes the only product. Thus pyridoxal phosphate is decomposed by light similarly to benzaldehyde, a benzoin condensation taking place in the absence of oxygen whereas a carboxylic acid is formed when oxygen is available. The mechanism of the photolysis of benzaldehyde has been discussed by Bäckström.[9] Pyridoxal phosphate differs from benzaldehyde in the great rapidity of its anærobic photolysis and in giving a dimer as the main product. From benzaldehyde the main products have been reported to be a

[2] Heyl, Luz, Harris, and Folkers, *J. Amer. Chem. Soc.*, 1951, **73**, 3434.
[3] Huff and Perlzweig, *J. Biol. Chem.*, 1944, **155**, 345.
[4] Dakin, *Amer. Chem. J.*, 1909, **42**, 486.
[5] Clutterbuck and Reuter, *J.*, 1935, 1467.
[6] Ide and Buek, " Organic Reactions," Vol. IV, p. 271.
[7] Eistert and Munder, *Chem. Ber.*, 1955, **88**, 215.
[8] Marsh and Stephen, *J.*, 1925, **127**, 1633.
[9] Bäckström, *Z. phys. Chem.*, 1934, **25**, 99.

tetramer and a trimer,[10] only small amounts of benzoin being obtained.[9] Benzoin has also been identified by Terenin[11] as a product of photochemical decomposition of benzaldehyde. Steric hindrance would probably suffice to prevent further condensation of the pyridoin derived from pyridoxal phosphate. Ultraviolet irradiation of acetaldehyde in aqueous solution has been reported[12] to give quantitative yields of acetoin.

Since pyridoxal phosphate is readily photolysed, it is important to protect this substance from strong light, particularly when in solution. Photolysis can be readily detected by the change in ultraviolet absorption spectrum of the substance. The pyridil is incapable of reactivating dissociated cysteinesulphinic acid decarboxylase, but does not compete with pyridoxal phosphate for this enzyme.[13]

Experimental

Photolysis of Pyridoxal Phosphate.—Pyridoxal phosphate monohydrate (0·53 g., 0·002 mole) was dissolved in 100 ml. of air-free water containing hydrochloric acid or sodium hydroxide to give solutions of pH 2, 4, 6, and 8 severally, which were exposed to bright summer sunlight for 1 hr. in stoppered Pyrex flasks, and their ultraviolet absorption spectra were measured at pH 7·0. The values tabulated did not change significantly after exposure to sunlight for a further hour.

	$E^{1\%}_{1\,cm.}$					
Wavelength	Pyridoxal		Photolysed solution			
(mμ)	phosphate	pH	2	4	6	8
288	30		199	195	192	209
329	99		82	86	69	58
387	202		50	44	52	82

A solution of pyridoxal phosphate monohydrate (5·3 g., 0·02 mole) in 250 ml. of air-free water containing 0·03 equivalent of sodium hydroxide was exposed to sunlight as in the previous experiment for 3 hr. It was then concentrated under reduced pressure to 50 ml. and run on to a column of Amberlite XE 97 cationic exchange resin (4 × 60 cm.). Water was passed through the column, the eluate being collected in 10 ml. fractions and the course of elution followed by making light-absorption measurements at 290 and 387 mμ at pH 7·0. Three products, isolated by evaporating appropriate fractions, corresponded to three spots observed when the photolysed solution was subjected to electrophoresis on filter paper. Two of these substances were present in amounts less than 20 mg. and were not examined further. The third substance which crystallised during evaporation was 5 : 5′-*bis*(*dihydroxyphosphinyloxymethyl*)-3 : 3′-*dihydroxy*-2 : 2′-*dimethyl*-4 : 4′-*pyridil monohydrate* (II) (4·6 g.), m. p. 235—240° (Found: C, 36·0; H, 4·25; N, 5·4; P, 11·7. $C_{16}H_{18}O_{12}N_2P_2,H_2O$ requires C, 36·4; H, 3·9; N, 5·5; P, 12·1%).

Pyridoxal phosphate had the following absorption spectra: in phosphate buffer (pH 7·0) $\lambda_{min.}$ 289—290 (ε 770), $\lambda_{max.}$ 329—330 (ε 2610), $\lambda_{min.}$ 338—340 (ε 2510), $\lambda_{max.}$ 387—388 mμ (ε 5320); in 0·1N-NaOH $\lambda_{min.}$ 288—290 (ε 700), $\lambda_{max.}$ 306—307 (ε 900), $\lambda_{min.}$ 318 (ε 820), $\lambda_{max.}$ 388 mμ (ε 6500); in 0·1N-HCl $\lambda_{min.}$ 247—248 (ε 800), $\lambda_{max.}$ 294—295 (ε 7350), $\lambda_{min.}$ 318—319 (ε 1120), $\lambda_{max.}$ 335 mμ (ε 1380).

The absorption spectra of the pyridil were: in phosphate buffer (pH 7·0) $\lambda_{max.}$ 288—289 (ε 5300), $\lambda_{min.}$ 320 (ε 600), $\lambda_{max.}$ 334—338 (ε 650), $\lambda_{min.}$ 356—360 (ε 620), $\lambda_{max.}$ 388—394 mμ (ε 700); in 0·1N-NaOH $\lambda_{min.}$ 317 (ε 1350), $\lambda_{max.}$ 390—394 mμ (ε 5300); in 0·1N-HCl $\lambda_{min.}$ 263 (ε 1270), $\lambda_{max.}$ 295 mμ (ε 8000).

Pyridil Oxime.—The pyridil (II) (500 mg.) in 2N-aqueous sodium carbonate (20 ml.) was boiled under nitrogen for 3 hr. with hydroxylamine hydrochloride (1·0 g.). The solution was brought to pH 3 with 2N-hydrochloric acid, and the precipitate collected and washed with boiling water (100 ml.), to give the *dioxime dihydrate* (350 mg.), decomp. 212—214° (Found: C, 35·2; H, 4·7; N, 10·2; P, 11·15. $C_{16}H_{20}O_{12}N_4P_2,2H_2O$ requires C, 35·5; H, 4·1; N, 10·4; P, 11·5%), $\lambda_{max.}$ 330 (ε 4620), $\lambda_{min.}$ 274 mμ (ε 2170) at pH 7·0.

4-*Pyridoxic Acid* 5-(*Dihydrogen Phosphate*) (I; X = CO_2H).—(a) *From the pyridil* (II). Hydrogen peroxide (1 ml. of 100-vol.) was added to the pyridil (II) (500 mg.) in 1·0N-sodium

[10] Ciamician and Silber, *Ber.*, 1909, **42**, 1386; Mascarelli, *Gazzetta*, 1906, **36**, II, 670.
[11] Terenin, *Acta Physicochim. U.R.S.S.*, 1940, **13**, 1.
[12] Dirscherl, *Z. physiol. Chem.*, 1930, **188**, 225.
[13] Davison, personal communication.

hydroxide (6 ml.). After 15 min. at room temperature 2N-hydrochloric acid (3 ml.) was added, to give 4-*pyridoxic acid* 5-(*dihydrogen phosphate*) which, recrystallised from water, had m. p. 203—205° (340 mg.) [Found: C, 37·1; H, 4·0; N, 5·4; P, 11·6%; equiv. (potentiometric titration), 94·6. $C_8H_{10}O_7NP$ requires C, 36·5; H, 3·8; N, 5·3; P, 11·7%; equiv., 94·6], $\lambda_{max.}$ 318 (ε 6720), $\lambda_{min.}$ 273—274 mμ (ε 1360) at pH 7·0.

(b) *From pyridoxamine* 5-(*dihydrogen phosphate*) (I; X = $CH_2{\cdot}NH_2$). Pyridoxamine phosphate (2·48 g.) was dissolved in 0·1N-sulphuric acid (50 ml.) and stirred with freshly precipitated manganese dioxide (10 g.). 10N-Sulphuric acid was added dropwise until the manganese dioxide had dissolved. The solution was kept at room temperature for 3 hr., the pH adjusted to 2·0 with sodium hydroxide, and the carboxylic acid filtered off and recrystallised (1·65 g.; m. p. 202—205°).

(c) *From pyridoxal* 5-(*dihydrogen phosphate*) (I; X = CHO). A solution of pyridoxal phosphate (2·65 g.) in 0·1N-sodium hydroxide (150 ml.) was exposed to a powerful ultraviolet lamp and a steady stream of oxygen was passed through it. After 6 hr. the peak at 388 mμ was replaced by one at 318—320 mμ. 2N-Hydrochloric acid (7·5 ml.) was added and, on concentration of the solution to *ca.* 50 ml., the pyridoxic acid phosphate (2·20 g.) crystallised; it had m. p. 201—205°.

Hydrolysis of 4-*Pyridoxic Acid* 5-(*Dihydrogen Phosphate*).—The phosphate (250 mg.) in concentrated aqueous hydrochloric acid (10 ml.) was boiled for 18 hr. under reflux. The solution was evaporated to dryness under reduced pressure and the residue treated with excess of saturated aqueous sodium hydrogen carbonate, whereupon the lactone of 4-carboxy-3-hydroxy-5-hydroxymethyl-2-methylpyridine was precipitated (135 mg.; m. p. 275—276°). This compound was identical with material prepared by the method of Heyl.[14]

Reduction of Pyridoxal 5-(*Dihydrogen Phosphate*) *with Sodium Borohydride*.—Sodium borohydride (500 mg.) was added to pyridoxal phosphate (530 mg.) in 0·5N-aqueous sodium hydroxide (10 ml.). When, after 5 min., the yellow solution had become colourless, its pH was adjusted to 5·0 with acetic acid and it was run on to a column of Amberlite XE 97 resin (2 × 30 cm.). The column was eluted with water and that fraction of the eluate absorbing ultraviolet light at 325 mμ was collected and evaporated to dryness under reduced pressure. The residue, freed from borate by repeated evaporation with methanol, was recrystallised from aqueous ethanol, to give pyridoxin 5-(dihydrogen phosphate) (310 mg.), m. p. 208—210°, identical with a sample made by deaminating pyridoxamine phosphate [1] (Found: C, 39·5; H, 5·0; N, 5·6; P, 12·4. Calc. for $C_8H_{12}O_6NP$: C, 38·5; H, 4·9; N, 5·6; P, 12·4%).

Reduction of the Pyridil (II) *with Sodium Borohydride*.—Sodium borohydride (500 mg.) was added to the pyridil (500 mg.) in 0·2N-sodium hydroxide (20 ml.). After 5 min. the mixture was worked up as in the previous experiment. Fractionation on XE 97 resin gave two fractions absorbing ultraviolet light at 325 mμ. They differed in the ease with which they could be freed from borate by evaporation with methanol, but once borate-free they had similar elementary analyses and behaved in the same way when oxidised with periodate (see below). Fraction A was eluted first. It was freed from borate by repeated evaporation with methanol, and the residue recrystallised from aqueous ethanol to give 5 : 5′-*bis*(*dihydroxyphosphinyloxymethyl*)-3 : 3′-*dihydroxy*-2 : 2′-*dimethyl*-4 : 4′-*hydropyridoin* (205 mg.), m. p. >300°. Fraction B was eluted from the column subsequently. After repeated evaporation with methanol (20 × 100 ml.), it was borate-free and recrystallised from aqueous ethanol to give the hydropyridoin (115 mg.), m. p. >300° [Found: (fraction A) C, 38·4; H, 4·4; N, 5·7; P, 13·1; (fraction B) C, 38·2; H, 4·3; N, 5·8; P, 12·9. $C_{16}H_{22}O_{12}N_2P_2$ requires C, 38·7; H, 4·5; N, 5·65; P, 12·5%).

Periodate Oxidations.—These were carried out with sodium metaperiodate in aqueous solution buffered to pH 4·0. The periodate remaining unconsumed after 4 hr. was estimated in the usual way by titrating, with standard arsenite, the iodine which it liberated from iodide. The products were examined by electrophoresis on filter paper (see below).

(*a*) Pyridoxal 5-(dihydrogen phosphate) used 0·15 mole of periodate per mole and gave small amounts of four products, the only one identified being 3 : 4-dihydroxy-2-methyl-5-pyridylmethyl dihydrogen phosphate (I; X = OH).

(*b*) The pyridil (II) used 1·0 mole of periodate per mole and gave 4-pyridoxic acid 5-(dihydrogen phosphate) (I; X = CO_2H) as the only product. From the pyridil (500 mg.), oxidised for 5 min. at room temperature with excess of sodium periodate, the crystalline carboxylic

[14] Heyl, *J. Amer. Chem. Soc.*, 1948, **70**, 3434.

acid (485 mg.), m. p. 202—205°, was obtained by adjusting the pH of the reaction mixture to 2·0.

(*c*) The hydropyridoin used 1·2 moles of periodate per mole. The main product was pyridoxal 5-(dihydrogen phosphate) which was isolated, in aqueous solution, by elution from the paper after electrophoresis of the reaction mixture; its identity was confirmed by its ultraviolet absorption. Other products corresponding to the substances formed by oxidation of pyridoxal phosphate itself were also found by electrophoresis on filter paper.

Electrophoresis on Filter Paper.—Electrophoresis was carried out on Whatman 3 mm. filter paper with a 0·067M-phosphate buffer at pH 7·0. Voltages of 300 v were applied to the paper (12 × 40 cm.) for 4—6 hr. The compounds were seen most easily under ultraviolet light after light spraying with 5N-ammonia. Relative rates of travel were as tabulated.

Substance	Rate of travel to anode	Appearance of spot
Pyridoxal 5-(dihydrogen phosphate)	1·0	Yellow fluorescent
Pyridoxamine 5-(dihydrogen phosphate)	0·6	Blue fluorescent
4-Pyridoxic acid 5-(dihydrogen phosphate)	1·1	Bright white fluorescent
3 : 4-Dihydroxy-2-methyl-5-pyridylmethyl dihydrogen phosphate	0·75	Absorbent
Pyridoxal	−0·1	Yellow weak fluorescent
4-Pyridoxic lactone	0·2	Bright white fluorescent
Pyridil (II)	1·0	Yellow, absorbent
Pyridoxal phosphate oxime	0·85	Blue fluorescent
Oxime of the pyridil	0·85	Absorbent

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