489. *An Unambiguous Synthesis of Codecarboxylase.*

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Phosphorylation of pyridoxal with dibenzyl chlorophosphonate gave pyridoxal-3 benzyl hydrogen phosphate (VII) which was converted into its hemiacetal (VIII) and then hydrogenated. The product was identical with the hemiacetal (I) of pyridoxal-3 dihydrogen phosphate described by other investigators and differed from codecarboxylase.

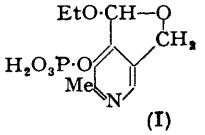
Unsuccessful attempts to synthesise codecarboxylase (III) through pyridoxal-3 benzyl ether (X) are described. The latter was prepared by oxidation of pyridoxine-3 benzyl ether and by benzylation of pyridoxal. Phosphorylation of the benzyl ether could not be achieved.

*OO-iso*Propylidenepyridoxine (XII; R = H) was prepared from pyridoxine and its structure was indicated by its ultra-violet absorption spectrum and proved by oxidation and hydrolysis to the lactone (XIV; R = H). It was phosphorylated with phosphoric oxide in phosphoric acid to (XII; R = PO_3H_2) from which the *iso*propylidene residue was removed by very mild acid hydrolysis. The resulting phosphate (XV) of pyridoxine was oxidised by known methods to the pyridoxal phosphate (III), identical in all respects with codecarboxylase.

CERTAIN amino-acids are decarboxylated to amines by micro-organisms. A coenzyme required in this transformation is called codecarboxylase. Its wide distribution throughout the animal and the plant kingdom suggests other functions, however, and it is now known that the same coenzyme is responsible for such processes as nitrogen transfer in amino-acids (transamination) and the synthesis of tryptophan from indole. Gunsalus, Bellamy, and Umbreit (*J. Biol. Chem.*, 1944, **155**, 685) showed that decarboxylation of tyrosine by dried cells of *Streptococcus fæcalis* was stimulated by the addition of pyridoxal and adenosine triphosphate or by an uncharacterised phosphorylation product of pyridoxal. This effect has been confirmed in the decarboxylation of other amino-acids (Baddiley and Gale, *Nature*, 1945, **155**, 727). The conclusion that codecarboxylase is a phosphate of pyridoxal was established by its synthesis in poor yield from pyridoxal and phosphoryl chloride (Gunsalus, Umbreit, Bellamy, and Foust, *J. Biol. Chem.*, 1945, **161**, 743). Although this synthesis established that the coenzyme was a monophosphate (Heyl, Harris, and Folkers, Abstr. 110th Meeting Amer. Chem. Soc., 1946, 34B), it did not reveal the position of the phosphate group on the pyridoxal molecule.

The first unambiguous synthesis of a phosphate of pyridoxal was that by Karrer and Viscontini (*Helv. Chim. Acta*, 1947, **30**, 52) who obtained the hemiacetal of the 3-(dihydrogen phosphate) (I) and hence the phosphate itself by the action of phosphoryl chloride on pyridoxal hemiacetal. These workers claimed high codecarboxylase activity for this substance (*ibid.*, p. 524) but others failed to confirm this and it was concluded that the 3-phosphate was not identical with the natural coenzyme (Gunsalus and Umbreit, *J. Biol. Chem.*, 1947, **170**, 415). Furthermore, the 3-phosphate gave an oxime which differed from that of " pyridoxal phosphate " (Heyl, Harris, and Folkers, *loc. cit.*; Heyl and Harris, *J. Amer. Chem. Soc.*, 1951, **73**, 3434). The demonstration of carbonyl function in the

coenzyme indicated that formulæ in which the phosphate group was attached to the 4-formyl residue were unlikely, although it did not eliminate such structures as (II) where a modified carbonyl function might be expected (Baddiley, Thain, and Rodwell, *Nature*, 1951, **167**, 556).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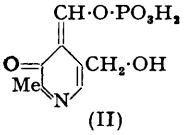
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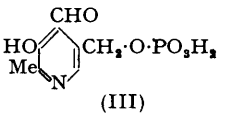
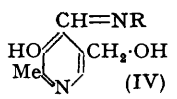

(III)

The work described in this paper aimed at an unambiguous synthesis of the alternative structure (III) for comparison with codecarboxylase. Since it was started, in 1949, other syntheses of " pyridoxal phosphate " have been announced from two independent groups of workers and leave little doubt that the coenzyme is correctly represented as (III). Folkers and his collaborators phosphorylated pyridoxal oxime (IV; R = OH) and claim to have deaminated the product with nitrous acid (Heyl, Harris, and Folkers, *loc. cit.*; Heyl, Luz, Harris, and Folkers, *J. Amer. Chem. Soc.*, 1951, **73**, 3430). The resulting phosphate which apparently showed high codecarboxylase activity contained an unsubstituted formyl residue at position 4 which could be replaced by hydroxyl by means of hydrogen peroxide (Heyl, Luz, and Harris, *ibid.*, p. 3437). Also, direct phosphorylation of pyridoxamine yielded pyridoxamine dihydrogen phosphate (V) which was readily oxidised to pyridoxal phosphate (Heyl, Luz, Harris, and Folkers, *ibid.*, p. 3436).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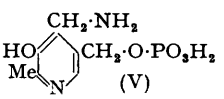
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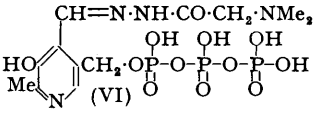
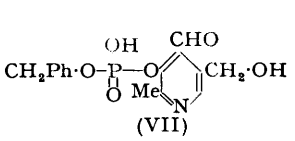

(VI)

Viscontini, Ebnöther, and Karrer (*Helv. Chim. Acta*, 1951, **34**, 1834, 2198) phosphorylated pyridoxal *N*-dimethylglycylhydrazone (IV; R = ·NH·CO·CH_2·NMe_2) with metaphosphoric acid, the product (VI) being converted into (III) by hydrolysis and treatment with nitrous acid.

These syntheses, together with the properties of the synthetic materials, leave little doubt that (III) correctly represents codecarboxylase. Nevertheless, no truly unambiguous synthesis has yet been described, since in those outlined above the phenolic hydroxyl group at position 3 remains unprotected throughout. The synthetic routes envisaged by us mainly involved protection by substitution of the phenolic hydroxyl group in either pyridoxine or pyridoxal derivatives, followed by phosphorylation, then removal of protecting groups. In our early experiments on these lines, however, the direct action of dibenzyl chlorophosphonate on pyridoxal was examined. Although the phenolic hydroxyl group was not protected during the reaction available evidence indicated that this reagent would not phosphorylate a phenol in pyridine solution. Phosphorylation of phenols with dibenzyl chlorophosphonate normally requires the phenol to be in the form of its sodium salt (Atherton, Openshaw, and Todd, *J.*, 1945, 382). In support, we have been able to show that both phenol and β-naphthol could be recovered unchanged after treatment with an excess of dibenzyl chlorophosphonate in pyridine. Under these conditions, however, pyridoxal gave an acidic product—a monobenzyl ester of pyridoxal phosphate—one benzyl group having been removed by quaternisation in the pyridine solution (cf. Baddiley, Clark, Michalski, and Todd, *J.*, 1949, 815). Its formulation as pyridoxal-3 benzyl hydrogen phosphate (VII) follows from its hydrolysis to pyridoxal and formation of an oxime and a hemiacetal (VIII). Hydrogenolysis of the benzyl group from the hemiacetal gave the acetal of pyridoxal-3 dihydrogen phosphate (I), identical with a sample prepared by Karrer and Viscontini's method (*loc. cit.*). This substance possessed no codecarboxylase activity.

From these rather unexpected observations it would seem that certain phenols may be phosphorylated with dibenzyl chlorophosphonate in pyridine solution. The enhanced reactivity in this respect of the 3-hydroxyl group of pyridoxal probably originates in the pyridine ring and is not an effect of the adjacent formyl group. This is indicated by the

observation that whereas salicylaldehyde is not phosphorylated under these conditions evidence was obtained that 3-hydroxypyridine is. Although no pure product was isolated from the latter a syrup was obtained which contained nitrogen and phosphorus and was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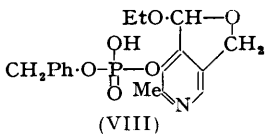
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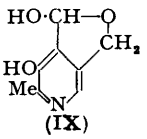
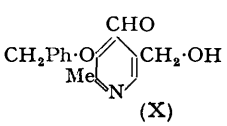

(IX)

readily soluble in chloroform. The indifference of the 5-hydroxymethyl group of pyridoxal towards this reagent, together with other observations on the unreactive nature of this hydroxyl group in related compounds described later in this paper, is consistent with the view that pyridoxal derivatives show a tendency to behave as cyclic compounds, *e.g.*, (IX) for pyridoxal itself (cf. Viscontini, Ebnöther, and Karrer, *Helv. Chim. Acta*, 1951, **34**, 2438).

Attention was then directed towards the preparation of pyridoxal-3 benzyl ether (X), from which the protecting benzyl group should be quite easy to remove by hydrogenolysis, subsequent to phosphorylation. Reaction between pyridoxal hemiacetal and benzyldimethylphenylammonium chloride (Leucotrope) readily yielded a benzyl ether. The acetal grouping in this compound was considerably more stable to acids than was pyridoxal hemiacetal, boiling with rather strong hydrochloric acid being necessary for the liberation of the free aldehyde. A crystalline oxime and 2 : 4-dinitrophenylhydrazone were prepared. The aldehyde was assigned the formula (X) both from its method of preparation and from its solubility in acids and insolubility in alkali. It did not give a ferric reaction and failed to couple with diazotised sulphanilic acid. It was converted into pyridoxine by hydrogenation in the presence of palladium–charcoal. Pyridoxal-3 benzyl ether may be prepared more conveniently by manganese dioxide oxidation of pyridoxine-3 benzyl ether (an intermediate in the synthesis of pyridoxine by Cohen's method, Jubilee Vol., Emil Barrell, 1946, p. 71; as we obtained pyridoxine for this investigation by Cohen's route, direct oxidation of the benzyl ether represented a considerable simplification). The oxidation product was purified by recrystallisation of its oxime, several recrystallisations being necessary to raise its melting point to that of the oxime prepared from the benzylation product of pyridoxal. This low melting point was doubtless caused by the presence of a small amount of the oxime of the isomeric 5-formyl compound.

Numerous attempts to phosphorylate pyridoxal-3 benzyl ether with phosphoryl chloride, diphenyl and dibenzyl chlorophosphonate, were without success. Whereas evidence of phosphorylation was obtained with diphenyl chlorophosphonate in pyridine, base hydrochloride being formed, the reaction product appeared to be very labile since only pyridoxal-3 benzyl ether was isolated. This suggests that the benzyl ether, like pyridoxal itself, tends to behave as a cyclic lactol. O^3-*p*-Nitrobenzoylpyridoxal is an analogous case since it was shown by Viscontini, Ebnöther, and Karrer (*Helv. Chim. Acta*, 1951, **34**, 2438) that with diphenyl chlorophosphonate this was converted into the exceptionally labile diphenyl phosphoric ester (XI).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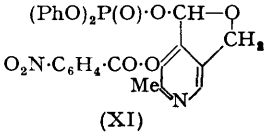
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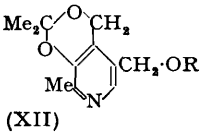
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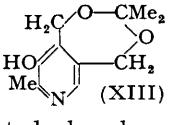
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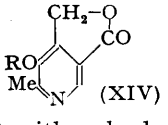
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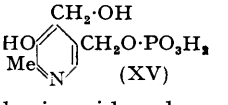
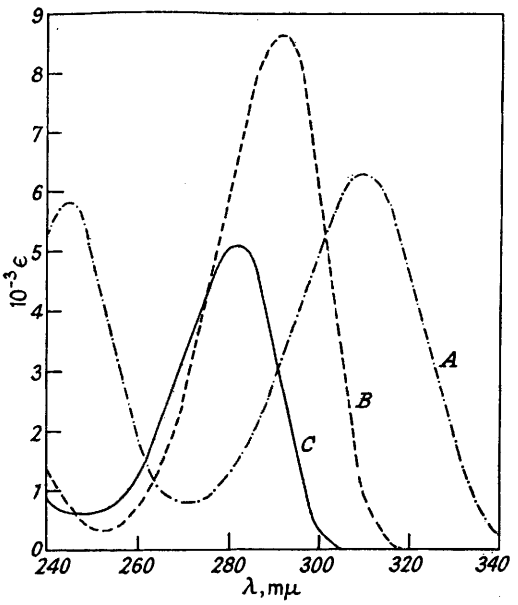

(XV)

Attempted phosphorylation of (X) with anhydrous phosphoric acid under conditions similar to those described in a different connection by Ferrell, Olcott, and Fraenkel-Conrat (*J. Amer. Chem. Soc.*, 1948, **70**, 2101) gave a black resin.

Pyridoxine and zinc chloride in hot acetone afforded a mono*iso*propylidene derivative in good yield. We are indebted to Dr. A. Cohen for informing us that he had obtained this compound by a similar method and has independent evidence of its structure. *OO-iso*Propylidenepyridoxine is very labile in dilute acid solution, giving pyridoxine and acetone. Of the two possible formulæ (XII; R = H) and (XIII) that in which the *iso*propylidene residue participates in a 6-membered ring (XII) seemed the more probable. This is supported by the failure of the substance, when freshly dissolved, to give a ferric reaction or a colour with diazotised sulphanilic acid. *iso*Propylidenepyridoxine exhibits a sharp absorption maximum at 2820 Å which is unchanged between pH 11·32 and 6·98. The curve (see Fig.) shows a close similarity to that obtained from pyridoxine itself in acid solution, *i.e.*, at pH 2·1 a single maximum at 2920 Å is reported (Stiller, Keresztesy, and Stevens, *ibid.*, 1939, **61**, 1237). Also pyridoxine-3 methyl ether exhibits a single absorption band at 2800 Å which is unaffected by pH change. In alkaline solution, however, pyridoxine shows two maxima as indicated in the Figure, the maximum at longer wave-length



A, *Pyridoxine*, pH 11·3.
B, *Pyridoxine in* 0·1N-HCl.
C, iso*Propylidenepyridoxine*, pH 7—11·3.

resulting from ionisation of the phenolic hydroxyl group. It is clear then that *iso*propylidenepyridoxine does not contain an ionisable phenolic hydroxyl group and so must be represented as (XII). Final proof of structure was obtained by oxidation with barium permanganate, followed by hydrolysis and acidification, whereby the lactone (XIV; R = H) was obtained, identical with that described by Harris, Stiller, and Folkers (*ibid.*, p. 1244). Methylation of this lactone gave the known 3-methyl ether (XIV; R = Me).

Hydrolysis of *iso*propylidenepyridoxine to pyridoxine was examined with some care since successful use of this derivative in a synthesis of " pyridoxal phosphate " depended on removal of the *iso*propylidene residue under conditions which would not cause hydrolysis of a phosphate ester. In the pyridoxine and pyridoxal series the phosphates are known to be labile to acids. The optimum conditions for this hydrolysis were determined readily by observing the change in the ultra-violet absorption spectrum at pH 11 as hydrolysis proceeded. At 20° in 0·01N-sulphuric acid very little pyridoxine was liberated in 2 hours, whereas at 100° the reaction had proceeded to 85% in 5 minutes and was complete in 10 minutes. Phosphoric esters in the pyridoxine series are mainly unaffected by such treatment.

Attempts were made to phosphorylate *iso*propylidenepyridoxine with phosphoryl chloride and diphenyl chlorophosphonate in pyridine and also in cold aqueous alkali, but in no case was a phosphoric ester produced. In those experiments where conditions had

been kept alkaline throughout, unchanged *iso*propylidene compound was recovered. The substance was also inert towards toluene-*p*-sulphonyl chloride and methanesulphonyl chloride in pyridine or aqueous alkali—the reason for this is not clear, particularly in view of the very ready formation of a *p*-nitrobenzoyl ester (XII; R = p-$O_2N{\cdot}C_6H_4{\cdot}CO$) in warm pyridine.

*iso*Propylidenepyridoxine and a solution of phosphoric oxide in anhydrous phosphoric acid (cf. Ferrell, Olcott, and Fraenkel-Conrat, *loc. cit.*; Wilson and Harris, *ibid.*, 1951, **73**, 4693) gave mixed phosphates. Paper chromatography showed as main component, *iso*propylidenepyridoxine phosphate (XII; R = PO_3H_2) with a small amount of 3-hydroxy-4-hydroxymethyl-2-methyl-5-pyridylmethyl phosphate (XV), presumably formed by hydrolysis of the former. These substances were identified by subjection of (XII; R = PO_3H_2) to a short acid hydrolysis and re-examination on paper. The product was identical with the slow-running contaminant. A second contaminant R_F 0·34 was also separated. Although this was not identified we consider that it is probably an isomer of (XV) in which the phosphate residue is attached to either the 3-hydroxyl or the 4-hydroxymethyl group. Furthermore, when the impure phosphate was run on a paper previously treated with borate buffer, only the spot corresponding to (XV) was retarded. Retardation of rate of flow in borate buffer was expected with this compound since it possesses free hydroxyl groups at position 3 and on the adjacent hydroxymethyl group and so, like pyridoxine itself, will form a borate complex which only moves slowly on paper at an alkaline pH.

The phosphate (XII; R = PO_3H_2) was purified by fractional precipitation of its barium salt. It was hydrolysed to (XV) by sulphuric acid and oxidised to a 5-formyl derivative by the method described by Heyl, Luz, Harris, and Folkers (*loc. cit.*). The product was identical in all respects with pyridoxal phosphate (III) prepared by the other synthetic routes and showed high codecarboxylase activity.

Experimental

Pyridoxal-3 Benzyl Hydrogen Phosphate.—Dibenzyl chlorophosphonate (prepared from 5 g. of dibenzyl phosphite) was added during 10 minutes to a solution of pyridoxal hydrochloride (1 g.) in anhydrous pyridine (*ca.* 20 c.c.) at about −40°. After 2—4 hours at room temperature water (5 c.c.) was added and solvent removed under reduced pressure. A solution of the syrupy residue in chloroform was extracted 3 times with water. The aqueous extract was evaporated to small volume under reduced pressure, then kept overnight. The crystalline solid which separated was filtered off, washed with a little cold water, then alcohol, and recrystallised from water. *Pyridoxal-3 benzyl hydrogen phosphate* formed small prisms, m. p. 202—203° (decomp.) (50—60%) (Found: C, 53·4; H, 5·0; N, 4·0; P, 9·1. $C_{15}H_{16}O_6NP$ requires C, 53·4; H, 4·8; N, 4·1; P, 9·2%), insoluble in hot alcohol, chloroform, etc., soluble in hot water, but sparingly soluble in cold water. It displaced carbon dioxide from a solution of sodium hydrogen carbonate.

Hydrolysis. The phosphate (0·2 g.) was heated at 100° in 5N-hydrochloric acid for 15 minutes. The cloudy solution was shaken with chloroform, and the aqueous fraction neutralised with sodium hydrogen carbonate. Sodium acetate (0·5 g.) and hydroxylamine hydrochloride (0·5 g.) were added and the solution warmed for a few minutes. Pyridoxal oxime was later deposited (85%), having m. p. 224° alone or mixed with an authentic sample, m. p. 225—226°.

Oxime. A solution of the phosphate (200 mg.), sodium acetate (200 mg.), and hydroxylamine hydrochloride (50 mg.) in water (7 c.c.) was heated at 100° during 10 minutes. After cooling to room temperature it was acidified to pH 2·5 with dilute sulphuric acid. The crystalline *oxime*, washed with water, then alcohol, and dried *in vacuo* at 80°, had m. p. 209·5° (203 mg., 97%) (Found: C, 51·1; H, 5·0; N, 8·2; P, 9·3. $C_{15}H_{17}O_6N_2P$ requires C, 51·0; H, 4·8; N, 8·0; P, 8·8%).

Ethyl Acetal (VIII) *of Pyridoxal-3 Benzyl Hydrogen Phosphate.*—Ethyl orthoformate (2·5 c.c.) was heated with a suspension of pyridoxal-3 benzyl hydrogen phosphate (266 mg.) in anhydrous ethyl alcohol (20 c.c.). Suspended solid dissolved slowly. Refluxing was continued for 4 hours, then the solution was kept at room temperature overnight. Solvent was removed under reduced pressure and the crystalline residue recrystallised from anhydrous alcohol. The *acetal* formed small needles, darkening at 166° and melting at 170° (decomp.) (84%) (Found: C, 55·7; H, 5·5; N, 3·4; OEt, 13·0. $C_{17}H_{20}O_6NP$ requires C, 55·8; H, 5·5; N, 3·8; OEt, 12·4%).

Hydrolysis. The acetal (87 mg.) was dissolved in water (3 c.c.) containing a drop of N-hydro-

chloric acid and the solution boiled for 5 minutes. Next morning crystals of pyridoxal-3 benzyl hydrogen phosphate were collected (64 mg., 87%), m. p. and mixed m. p. 203° (decomp.).

Ethyl Acetal of Pyridoxal-3 Dihydrogen Phosphate.—The acetal of pyridoxal-3 benzyl hydrogen phosphate (300 mg.) was hydrogenated in alcohol (*ca.* 50 c.c.) at room temp./1 atm. in the presence of palladium oxide. The calculated amount of hydrogen was absorbed rapidly. After filtration and evaporation under reduced pressure, the remaining brittle resin was triturated with alcohol, giving a fine white powder which charred at about 200° (no m. p.) (Found : C, 43·6; H, 5·3; N, 4·8; P, 11·7; OEt, 17·1. Calc. for $C_{10}H_{14}O_6NP$: C, 43·6; H, 5·1; N, 5·1; P, 11·3; OEt, 16·4%). The product did not possess any activity as tyrosine codecarboxylase.

2 : 4-*Dinitrophenylhydrazone of Pyridoxal-3 Dihydrogen Phosphate.*—To a solution of the above acetal in a little water was added a hot solution of 2 : 4-dinitrophenylhydrazine in N-hydrochloric acid. After boiling for 1 minute the mixture was cooled and the orange-yellow plates were filtered off. The 2 : 4-*dinitrophenylhydrazone* was washed well with water, alcohol, and ether, then dried in a desiccator over sulphuric acid (Found : C, 39·4; H, 3·4. $C_{14}H_{14}O_9N_5P$ requires C, 39·2; H, 3·3%). *X*-Ray powder photographs of this dinitrophenylhydrazone were indistinguishable from those of the material prepared from a sample of Karrer and Viscontini's substance (*loc. cit.*).

A very small sample of pyridoxal-3 dihydrogen phosphate was converted in the usual way into an oxime, m. p. 212° (decomp.). Heyl and Harris (*J. Amer. Chem. Soc.*, 1951, **73**, 3434) report m. p. 210—211° (decomp.) for this substance. The quantity obtained was insufficient for analysis.

Hemiacetal of Pyridoxal-3 Benzyl Ether.—To pyridoxal hemiacetal hydrochloride (2 g.) dissolved in the minimum quantity of anhydrous alcohol was added a solution of sodium (0·4 g.) in alcohol, followed by benzyldimethylphenylammonium chloride (2·3 g.) in the minimum quantity of anhydrous alcohol. The mixture was run into boiling xylene (20 c.c.) with continuous stirring in an atmosphere of nitrogen. The experiment was so arranged that all the alcohol and a little xylene distilled off during 90 minutes. The solution was filtered, cooled, then washed successively with sodium hydrogen carbonate solution, 2N-sodium hydroxide, and water, and finally dried (Na_2SO_4). Xylene and dimethylaniline were removed by distillation under reduced pressure and the residue solidified on being rubbed with a little petroleum (b. p. 40—60°). The *hemiacetal* (1·9 g., 78%) was purified by recrystallisation from ether–light petroleum (b. p. 40—60°) or by sublimation in a short-path still at 80°/10^{-5} mm., being obtained as colourless needles, m. p. 48° (Found : C, 71·4; H, 6·5; N, 4·9; OEt, 16·7. $C_{17}H_{19}O_3N$ requires C, 71·6; H, 6·7; N, 4·9; OEt, 15·8%).

Pyridoxal-3 Benzyl Ether (*Method* I).—The above hemiacetal (3·9 g.) was dissolved in 5N-hydrochloric acid (4·1 c.c.), the solution diluted to 10 c.c. with water, and heated in boiling water for 30 minutes. The solution was evaporated under reduced pressure to 5 c.c., then adjusted to pH 8 with sodium hydrogen carbonate solution, and the product extracted three times with ether. The combined extracts were dried (Na_2SO_4) and evaporated. The solid residue was recrystallised from ethyl acetate. Pyridoxal-3 benzyl ether (2 g., 60%) had m. p. 151—152°, undepressed on mixing with a sample prepared by the method described below.

Oxime of Pyridoxal-3 Benzyl Ether (*Method* I).—The hemiacetal (0·5 g.) was dissolved in the minimum quantity of alcohol, 1·12N-hydrochloric acid (20·7 c.c., 1·66 mols.) added, and the solution refluxed for 1 hour. 0·91N-Sodium hydroxide (8·46 c.c.) was added and the resulting precipitate redissolved by addition of alcohol. Hydroxylamine hydrochloride (0·19 g.) was added and the solution heated under reflux for 1 hour. Excess of alkali was neutralised with hydrochloric acid, and the precipitate filtered off. The impure oxime was dissolved in alcohol, and the solution decolorised with charcoal and concentrated. Addition of water precipitated the oxime which was purified by two recrystallisations from ethyl acetate. It softened at 157° and had m. p. 161—162°, undepressed when mixed with a sample prepared as described below.

2 : 4-*Dinitrophenylhydrazone of Pyridoxal-3 Benzyl Ether.*—The hemiacetal was dissolved in a little methanol and an excess of 5N-hydrochloric acid added. The solution was boiled for 5 minutes and a solution of 2 : 4-dinitrophenylhydrazine hydrochloride in aqueous methanol was added. Boiling was continued for a few minutes, then the solution was cooled and scratched. A pale-yellow solid was deposited. This was filtered off, washed with methanol and water, then recrystallised from aqueous dioxan. After two recrystallisations the product was considerably deeper in colour than the original pale-yellow material and whereas the latter contained ionic halogen the recrystallised product was halogen-free. The pale-yellow material is probably the hydrochloride of the 2 : 4-dinitrophenylhydrazone while the darker substance is the free base. The pure *dinitrophenylhydrazone* had m. p. 181—182°, undepressed in admixture with

the 2 : 4-dinitrophenylhydrazone, m. p. 181—182°, prepared from pyridoxal-3 benzyl ether by the second method (Found : N, 15·8. $C_{21}H_{19}O_6N_5$ requires N, 16·0%).

Oxime of Pyridoxal-3 Benzyl Ether (*Method* II).—Manganese dioxide (1·77 g. of 83·1% purity) was added to a solution of pyridoxine-3 benzyl ether hydrochloride (5 g.) in water (75 c.c.). The mixture was stirred vigorously during the dropwise addition of concentrated sulphuric acid (0·91 c.c.) and then kept at 70° in a water-bath, also with good stirring, until the pH had reached about 5 (approx. 90 minutes). A small amount of undissolved inorganic material was removed by filtration and sodium acetate (4·15 g.) added to the filtrate. The precipitate which formed was redissolved in alcohol, and hydroxylamine hydrochloride (1·75 g.) added to the solution which was then heated under reflux for 1 hour. On addition of water (100 c.c.) a precipitate appeared. This was filtered off, washed with water, and dissolved in hot alcohol, and the solution decolorised with charcoal, concentrated, and cooled. The *oxime* crystallised as fine needles, m. p. 138—142°, raised to 150—155° after three recrystallisations from aqueous alcohol. Two further crystallisations from ethyl acetate raised the m. p. to 161—162°, the same as that given by the oxime prepared by Method I (Yield 1—2 g.) (Found : C, 66·4; H, 5·9; N, 10·2. $C_{15}H_{16}O_3N_2$ requires C, 66·4; H, 5·9; N, 10·3%).

Pyridoxal-3 Benzyl Ether (*Method* II).—Sodium nitrite (0·65 g., 3 mols.) in a little water was added slowly, with shaking, to a solution of the oxime of pyridoxal benzyl ether (0·85 g.) in acetic acid. After 30 minutes at room temperature the solution was warmed to 30° for 1 hour, then excess of nitrous acid was decomposed by heating the mixture at 60° for a short time. Acetic acid and water were removed by evaporation under reduced pressure, and the residue was dissolved in N-hydrochloric acid (6 c.c.). The pH was adjusted to 8 with sodium hydrogen carbonate solution, and the product extracted three times with ether. The extracts were combined, dried (Na_2SO_4), and evaporated, leaving a residue of *pyridoxal-3 benzyl ether* which, after recrystallisation from ethyl acetate, had m. p. 151—152° (0·5 g., 62%) (Found : N, 5·3. $C_{15}H_{15}O_3N$ requires N, 5·5%).

iso*Propylidenepyridoxine*.—Pyridoxine hydrochloride (10 g.; dried for 5 hours at 110°/0·1 mm. over phosphoric oxide) was added to a filtered solution of pure, fused zinc chloride (33 g.) in anhydrous acetone (330 c.c.). After 5 hours' boiling under reflux with exclusion of moisture the cloudy solution was kept at room temperature for 12 hours. Approximately two-thirds of the acetone were removed under reduced pressure and the remaining solution poured into a warm (40°) solution of barium hydroxide (90 g. of octahydrate) in water (*ca.* 600 c.c.). The mixture was cooled rapidly to room temperature and carbon dioxide passed in to remove alkalinity. Zinc and barium carbonates were filtered off and washed thoroughly with alternate lots of boiling methanol and water (*ca.* 500 c.c. of each). The combined filtrate and washings were evaporated to dryness under reduced pressure below 40° and the dried residue was extracted continuously with dry acetone for 5 hours. After evaporation of the acetone the residue was dissolved in 90% methanol (200 c.c.), made alkaline with ammonia, and saturated with hydrogen sulphide. A small amount of precipitated zinc sulphide was removed by filtration through silica, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 50% methanol, and the solution decolorised with charcoal and evaporated. iso*Propylidenepyridoxine* recrystallised from boiling water as needles, m. p. 108—109° (6·3 g., 63%) (Found : C, 63·4; H, 7·2; N, 6·6. $C_{11}H_{15}O_3N$ requires C, 63·4; H, 7·2; N, 6·7%). In a phosphate buffer at pH 7 it showed an absorption maximum at 282 mμ ($\varepsilon = 5080$). In a glycine–sodium hydroxide buffer at pH 11·3 it showed a maximum at 282 mμ ($\varepsilon = 5170$).

3-*Hydroxy*-4-*hydroxymethyl*-2-*methylpyridine*-5-*carboxylic* 4-*Lactone* (XIV; R = H).—To a solution of *iso*propylidenepyridoxine (0·3 g.) in water (20 c.c.) was added barium permanganate (0·36 g.) in water (15 c.c.) with good stirring during 30 minutes. The precipitated manganese dioxide was removed by centrifugation and washed with water, and the combined supernatant liquors were evaporated to dryness three times with water to remove ammonia. The residue was dissolved in 0·5N-sulphuric acid (10 c.c.), and the solution kept at room temperature for 24 hours, then heated on a steam-bath for 30 minutes to ensure lactonisation. The calculated amount of barium hydroxide solution was added to the cooled solution, barium sulphate removed by centrifugation, and the sediment washed with water. The combined supernatant layer and washings were concentrated under reduced pressure and chilled. The lactone separated as fine needles, which after recrystallisation from alcohol had m. p. 268—269° (60 mg.) (Found : C, 57·9; H, 4·5; N, 8·5. Calc. for $C_8H_7O_3N$: C, 58·2; H, 4·2; N, 8·5%). Harris, Stiller, and Folkers (*loc. cit.*) report m. p. 272—273°.

4-*Hydroxymethyl*-3-*methoxy*-2-*methylpyridine*-5-*carboxylic* 4-*Lactone* (XIV; R = Me).—This was prepared by methylation of the above lactone according to the method of Harris, Stiller,

and Folkers (*loc. cit.*), who record m. p. 108—109°. Our substance had m. p. 105—106° (Found : C, 60·7; H, 5·4; N, 8·0. Calc. for $C_9H_9O_3N$: C, 60·3; H, 5·0; N, 7·8%).

Hydrolysis of iso*Propylidenepyridoxine.*—In the determination of optimum hydrolysis conditions for *iso*propylidenepyridoxine use was made of the fact that it does not absorb light at 309 mμ at pH 11·3, whereas the hydrolysis product, pyridoxine, shows a maximum at that wavelength. The *iso*propylidene compound (0·0522 g.) was dissolved in 0·01N-sulphuric acid (200 c.c.), and the solution heated under reflux in a boiling-water bath. Portions were removed from time to time and cooled rapidly, then 2-c.c. aliquots were each diluted to 25 c.c. with glycine–sodium hydroxide buffer (pH 11·3), thereby giving a 0·0001M-solution. From the values of the extinction coefficients given by these samples it was calculated that the *iso*propylidene residue was 85% hydrolysed in 5 minutes at 100° under these conditions and that hydrolysis was complete in 10 minutes.

O^3-p-*Nitrobenzoyl*-OO-iso*propylidenepyridoxine.*—*p*-Nitrobenzoyl chloride (0·23 g.) was added to a solution of *iso*propylidenepyridoxine (0·25 g.) in anhydrous pyridine (3 c.c.), and the mixture refluxed for 5 minutes. After cooling, water (10 c.c.) was added and the solid which separated was filtered off, washed with water, and recrystallised from light petroleum (b. p. 80—100°). The p-*nitrobenzoyl* derivative had m. p. 178—179° (Found : C, 59·2; H, 4·9; N, 8·0. $C_{17}H_{16}O_6N_2$ requires C, 59·3; H, 4·7; N, 8·1%).

Phosphorylation of iso*Propylidenepyridoxine.*—Phosphoric oxide (9·3 g.) was weighed rapidly into phosphoric acid (13·2 g. of 88%) and the mixture heated with stirring until a clear solution was obtained. After the whole had cooled to room temperature in a desiccator, powdered *iso*propylidenepyridoxine (3 g.) was added with stirring and the mixture kept in a desiccator over phosphoric oxide, being stirred several times daily during the first 3 days until a clear, viscous syrup was obtained. After a further 4 days at room temperature the syrup was stirred vigorously with dioxan (30 c.c.). The resulting mobile liquid was added dropwise to a suspension of finely powdered barium hydroxide (90 g. of octahydrate) and crushed ice (200 g.) in water (150 c.c.) contained in a large mortar. The mixture was ground vigorously throughout the addition, more ice being added as required. Excess of barium hydroxide was neutralised by passing in carbon dioxide, and the precipitate removed by centrifugation. After thorough washing of the sediment with large amounts of boiling water, alternate trituration and filtration being the most convenient method, the combined supernatant liquids and washings were concentrated under reduced pressure to about 250 c.c. A small precipitate (*ca.* 150 mg.) was removed by centrifugation and the supernatant liquor diluted with 3 vols. of alcohol. On storage at 0° the impure phosphate was deposited. It was centrifuged, washed with alcohol, then ether, and dried in a desiccator (2·1 g. of white powder).

A sample of the impure phosphate (0·5 mg.) was dissolved in water (0·2 c.c.), and ammonium sulphate solution added to precipitate barium. Barium sulphate was removed by centrifugation and the solution evaporated to dryness in a desiccator (vacuum). The residue was dissolved in water (0·05 c.c.) and chromatographed on Whatman No. 1 paper, with *n*-propanol–ammonia–water, followed by the perchloric acid–molybdate spray of Hanes and Isherwood (*Nature*, 1949, **164**, 1107). Alternatively ultra-violet light (Mineralight V.41 lamp) was also used for locating spots. Pyridoxine and pyridoxal derivatives appear deep purple or vivid blue when examined in this way. No special temperature control was employed and unwashed paper was quite satisfactory. The R_F values given are for room temperature and represent averages taken from several experiments. The following spots were observed : (1) a strong spot, R_F 0·71 (*iso*propylidenepyridoxine phosphate), (2) a spot, R_F 0·34 (probably an isomeric pyridoxine phosphate), (3) a weak spot, R_F 0·21 [pyridoxine phosphate (XV)].

Identification of the spots was established by hydrolysis and re-examination of a sample. The impure barium salt (4·2 mg.) was treated with 0·01N-sulphuric acid (10 c.c.) and precipitated barium sulphate removed by centrifugation. The solution was boiled for 7 minutes, then cooled rapidly, and a slight excess of barium hydroxide solution added. Excess of barium hydroxide was removed by carbon dioxide, the solution being boiled for 1 minute, then centrifuged. The resulting solution was prepared for chromatography as described above. The strong spot, R_F 0·71, disappeared after this treatment whereas the spot, R_F 0·21, had increased considerably in intensity and area, indicating conversion of the fast-running into the slower substance.

When impure *iso*propylidenepyridoxine phosphate was run on a paper which had been treated previously with borate buffer solution and then dried, the fast-running main component (R_F 0·71) moved slightly faster (R_F 0·86) while the slowest component was reduced in R_F from 0·21 to 0·14.

Purification through the Barium Salt.—The impure barium salt (0·5 g.) was dissolved in the minimum amount of water (*ca.* 100 c.c.), and alcohol added with stirring to the first permanent opalescence. After 5—10 minutes the precipitate was removed by centrifugation, washed with alcohol and ether, and dried. The process was repeated on the supernatant liquid giving, in all, 4 fractions. Examination by paper chromatography showed that the first two fractions, which comprised about 70% of the original material, gave a strong spot, R_F 0·71, and two very weak, slow-developing spots, R_F 0·34 and 0·21. The last two fractions contained all components in approximately equal proportions. The mother-liquors, on evaporation, left a small residue which gave a spot, R_F 0·48, on paper; this was discarded. The first two fractions were combined and reprecipitated, giving pure *barium* iso*propylidenepyridoxine phosphate* (about 0·3 g.) (Found: N, 3·4; P, 7·0; Ba, 32·7. $C_{11}H_{14}O_6NPBa$ requires N, 3·3; P, 7·3; Ba, 32·4%). Analytical figures for carbon and hydrogen for this substance were rather low and unreliable, widely different results being obtained on repeated analysis of the same sample, probably owing to the well-known difficulty experienced in combustion of barium salts of phosphates.

4-*Formyl*-3-*hydroxy*-2-*methyl*-5-*pyridylmethyl Dihydrogen Phosphate* (*Pyridoxal Phosphate*) (III).—The above barium salt (0·1 g.) was heated with 0·907N-sulphuric acid (0·81 c.c.) and water (1·72 c.c.) for 8 minutes at the b. p. After rapid cooling, a slight excess of barium hydroxide solution was added and excess of barium removed by carbon dioxide and boiling for 1 minute. The precipitate was removed by centrifugation, then washed with water, and the combined supernatant liquid and washings were evaporated under reduced pressure to about 4 c.c. Alcohol (4 vols.) and ether (4 vols.) were added and the precipitated pyridoxine phosphate was separated by centrifugation and washed with alcohol–ether and dried in a desiccator (0·806 g., 89%). Examination of this substance on paper showed that it was homogeneous (R_F 0·21).

Oxidation to the aldehyde was accomplished by the method given by Heyl, Luz, Harris, and Folkers (*loc. cit.*), and the product isolated by precipitation of the barium salt with alcohol or by conversion into its oxime. The oxime was a sparingly soluble crystalline substance which decomposed between 180° and 210° with blackening but without melting (Found: C, 36·0; H, 4·5; N, 10·5; P, 12.2 Calc. for $C_8H_{11}O_6N_2P$: C, 36·6; H, 4·2; N, 10·7; P, 11·8%). The American workers report a m. p. varying between 218° and 230° for the oxime of pyridoxal phosphate but a sample prepared by us from pyridoxal phosphate synthesised by the method of Viscontini, Ebnöther, and Karrer (*loc. cit.*) decomposed without melting between 180° and 195°. No depression was observed on admixture of the two samples and they were indistinguishable on paper chromatography in *n*-propyl alcohol–ammonia (R_F 0·25).

A sample of the barium salt of the free aldehyde, when examined on paper in the same way showed a strong spot (R_F 0·35), indistinguishable from that given by authentic pyridoxal phosphate. Some unoxidised pyridoxine phosphate was also present (R_F 0·21). Pyridoxal phosphate was visible as a bright yellow spot on the paper before spraying with the perchloric acid–molybdate. It possessed the same activity, within the limits of experimental error, as that of Viscontini, Ebnöther, and Karrer in the activation of tyrosine apodecarboxylase.

We are indebted to Dr. E. F. Gale for the enzyme tests. Sincere thanks are also offered to Dr. A. Cohen and his colleagues for unpublished information on the synthesis of pyridoxine. The *X*-ray powder photograph was obtained through the courtesy of Professor A. R. Todd, F.R.S. Gifts of chemicals from Roche Products Ltd. and Lederle Laboratories are gratefully acknowledged. This work was carried out during the tenure of studentships from the Medical Research Council and the Department of Scientific and Industrial Research (to A. P. M.).

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[*Received, March 4th*, 1952.]