Reaction of Phenylmagnesium Bromide with Cycloöctatetraene.—A solution of phenylmagnesium bromide was prepared from 31.4 g. (0.20 mole) of bromobenzene and 4.86 g. of magnesium in 90 ml. of dry ether. Cycloöctatetraene (20.8 g., 0.20 mole) was added, and the homogeneous solution that was formed was stirred and heated to a bath temperature of 100°. After 1 hour the brown liquid mixture had changed to a bright yellow solid; stirring was discontinued, and heating at 100° was continued for 1 hour longer. The mixture was cooled with ice, and 125 ml. of a cold, saturated solution of ammonium chloride in water was added with stirring. The yellow ethereal layer was separated, and the aqueous layer was extracted with 50 ml. of ether. The combined ethereal solutions were washed with water, dried over magnesium sulfate, and concentrated. Two fractionations of the low boiling material through a semi-micro column separated fraction 1, 16.3 g., b.p. 73–78° (89 mm.), $n^{25}D$ 1.5268. This fraction was determined to contain 2.4% of bromobenzene by quantitative analysis for bromine, 71% (56% recovery) of cycloöctatetraene by colorimetric analysis,[16] and 27% or 4.4 g. of cyclooctatrienes (42% yield) by difference. Extraction with 20% silver nitrate by the procedure previously described separated 8.44 g. (41% recovery) of cycloöctatetraene, b.p. 74–75° (91 mm.), $n^{25}D$ 1.5322, and 3.33 g. (31%) of cyclooctatrienes, b.p. 78–80° (90 mm.), $n^{25}D$ 1.5132. Treatment with potassium *t*-butoxide in *t*-butyl alcohol yielded 2.18 g. of crude 1,3,5-cycloöctatriene, b.p. 80–82° (92 mm.), $n^{25}D$ 1.5222, which was identified by conversion to the maleic anhydride adduct, m.p. 143–144°.

The high boiling residue from which fraction 1 was separated crystallized on cooling. It was recrystallized from ethanol and sublimed at 0.5 mm. with a heating block temperature of 75–80°, and yielded 5.8 g. of biphenyl, m.p. 69.5–70.5°. An additional 1.2 g. was obtained from the ethanol mother liquor by sublimation, making the total yield 7.0 g. (45%). The residue from the sublimation was a dark orange resin.

A control experiment was conducted to determine what proportion of the biphenyl isolated from the reaction of phenylmagnesium bromide with cycloöctatetraene could be derived from the Grignard reagent without participation of cycloöctatetraene in the reaction. A solution of phenylmagnesium bromide was prepared from 31.4 g. of bromobenzene and 4.86 g. of magnesium in 90 ml. of ether. Ethylcyclohexane (20.8 g.) was added and the mixture (two layers) was stirred and heated to a bath temperature of 100°, while 30 ml. of ether distilled. Stirring and heating at 100° were continued for 2 hours, after which the mixture was cooled, hydrolyzed, and the product was isolated in the same manner as the products obtained from phenylmagnesium bromide and cycloöctatetraene. The yield of biphenyl, m.p. 68–70°, was 1.02 g. (6.6%).

CAMBRIDGE, MASS. RECEIVED OCTOBER 30, 1950

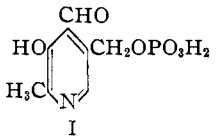
[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF MERCK & CO., INC.]

Phosphates of the Vitamin B_6 Group. I. The Structure of Codecarboxylase

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

Evidence which is presented indicates that codecarboxylase is the monophosphoric acid ester of the 5-hydroxymethyl group of pyridoxal. It has been isolated in pure form as the oxime and as the O-methyloxime. The oxime of the betaine of 1,2-dimethyl-3-hydroxy-4-formyl-5-pyridylmethylphosphoric acid has also been obtained. The calcium salt of 2-methyl-3-hydroxy-4-hydroxymethyl-5-pyridylmethylphosphoric acid has been obtained after phosphorylation of pyridoxine with phosphorus oxychloride in the presence of water.

Codecarboxylase has been synthesized, and evidence is now available to support the conclusion that it is the monophosphoric acid ester, I, of the 5-hydroxymethyl group of pyridoxal.



I

Codecarboxylase[1] was found to be a phosphorylated derivative of pyridoxal; later its activity as a cotransaminase[2] in the synthesis of amino acids was discovered. Pyridoxal phosphate was originally prepared by the action of adenosine triphosphate on pyridoxal.[1] It was prepared later by the action of phosphorus oxychloride on pyridoxal in the presence of water.[3]

Codecarboxylase is formed in low yield by phosphorylation of pyridoxal in aqueous solution with phosphorus oxychloride, and has been isolated as a calcium salt by direct neutralization of the phosphorylation mixture with calcium carbonate. The calcium phosphate is precipitated, leaving codecarboxylase in solution. The addition of ethyl alcohol to the solution precipitates the calcium salt of the coenzyme. This material has been estimated by assay to contain 50–80% of co decarboxylase.[4]

Codecarboxylase has been characterized as a pure crystalline oxime,[5] for which the formula $C_8H_{11}N_2O_6P$ was demonstrated. The same oxime was prepared by phosphorylation of pyridoxal oxime with phosphorus oxychloride in aqueous solution. The formula for the oxime also established the formula $C_8H_{10}NO_6P$ for codecarboxylase itself, showing it to be a condensation product of pyridoxal and phosphoric acid with the elimination of one molecule of water. The analytical data preclude the presence of a phosphoric acid ester group on either the 6-position or the 2-methyl group.

Positive ferric chloride color tests for both the calcium salt and the oxime of codecarboxylase show the presence of a free phenolic hydroxyl group. This result was substantiated by the synthesis of 3-pyridoxalphosphoric acid oxime, which was not identical with codecarboxylase oxime.[5,6] The formyl group (in hemi-acetal linkage with the 5-hydroxymethyl group) was eliminated as the location of the phosphate group both by the formation of the oxime without elimination of the phosphate

(1) Gunsalus, Bellamy and Umbreit, *J. Biol. Chem.*, **155**, 685 (1944).

(2) Lichstein, Gunsalus and Umbreit, *ibid.*, **161**, 311 (1945).

(3) Gunsalus, Umbreit, Bellamy and Faust, *ibid.*, **161**, 743 (1945).

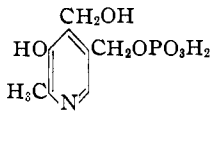
(4) Umbreit, Bellamy and Gunsalus, *Arch. Biochem.*, **7**, 185 (1945). We are indebted to Drs. Gunsalus and Umbreit for assaying our preparations.

(5) Heyl, Harris and Folkers, Abstracts, American Chemical Society, 110th Meeting, Chicago, 35B (1946).

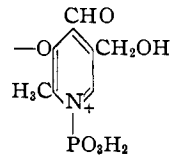
(6) Heyl and Harris, THIS JOURNAL, **73**, 3434 (1951).

group and by the oxidation of pyridoxine phosphate (II), by means of manganese dioxide and sulfuric acid, to codecarboxylase, which was isolated as the oxime. Pyridoxine phosphate was prepared by phosphorylation of pyridoxine in aqueous solution with phosphorus oxychloride.

These data narrow the possible structures for codecarboxylase to I and III. The original reasons



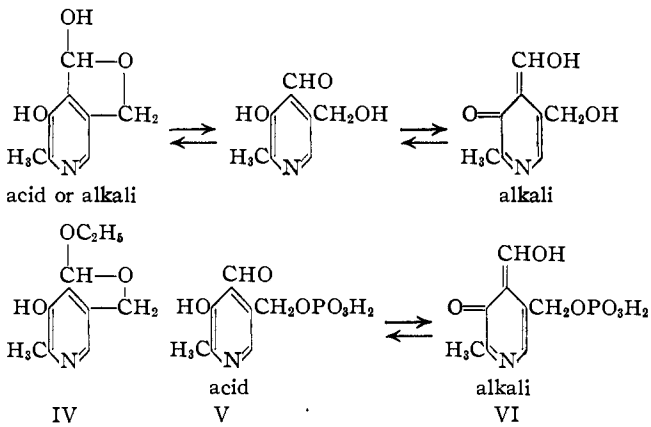
II



III

for considering structure III were the unusual absorption spectrum,[3,7] Fig. 1, of codecarboxylase in alkaline solution and also the lack of a color test with dichloroquinone chloroimide in alkaline solutions. Other evidence is now available which explains these seeming anomalies.

Pyridoxal and codecarboxylase, which are colorless in acid solution, form bright yellow solutions at alkaline *p*H's. They have similar absorption spectra in acid solution (Fig. 2) but have very different ones in alkaline solution (Fig. 1). The structure of the phosphate, in which the cyclic hemi-acetal grouping is impossible, is changed more in alkali than is that of pyridoxal itself. These changes may be represented by the following scheme of reactions.



IV V VI

Pyridoxal monoethylacetal (IV) shows the typical ultraviolet spectrum of 3-hydroxypyridine[8] in alkali (Fig. 1), while the spectrum of pyridoxal shows a tendency to shift to the longer wave lengths and that of pyridoxal phosphate is almost completely shifted to a band of 3900 Å. The absorption spectra of all three of these compounds in acid solution are quite similar (Fig. 2). The changes in absorption spectra in alkaline solution are apparently due to the presence of both the free 4-formyl group and the free 3-hydroxy group.[6,9]

The chloroimide phenol test,[10] the absence of which has been taken as an indication that co-

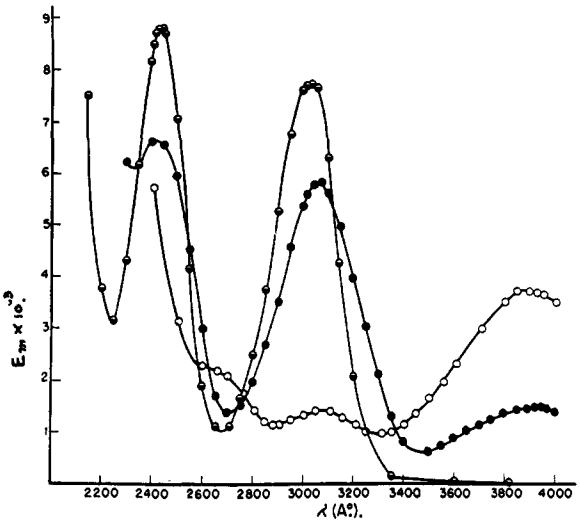
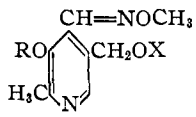


Fig. 1.—Absorption spectra at *p*H 11.0: ●, pyridoxal; ○, codecarboxylase; ◒, pyridoxal monoethylacetal.

decarboxylase is 3-pyridoxalphosphoric acid,[11,12] depends on the directive influence of a free phenolic group for coupling in position 6, and may be in-



VII, R = H, X = H
VIII, R = H, X = PO_3H_2
IX, R = CCH_3, X = H
O

fluenced by other substituents in the ring.[13] Structure VI may explain why no color is given with this reagent in alkaline solutions.

The O-methyloximes of both pyridoxal (VII) and codecarboxylase (VIII) have been prepared. Acetylation of the former in aqueous solution yields the 3-acetoxy derivative (IX).

Pyridoxal monomethylacetal was methylated to form the monomethyl acetal of pyridoxal methiodide (X), which was hydrolyzed and treated with silver chloride to form pyridoxal methochloride (XI). The latter was phosphorylated with phosphorus oxychloride in water to yield the betaine of 1,2-dimethyl-3-hydroxy-4-formyl-5-pyridylmethylphosphoric acid (XII), which was obtained pure as the oxime (XIII).

Experimental[14]

Calcium Codecarboxylase (Calcium Salt of 2-Methyl-3-hydroxy-4-formyl-5-pyridylmethylphosphoric Acid) (I).—To a solution of 5.0 g. of pyridoxal hydrochloride in 35 ml. of water, 25 ml. of phosphorus oxychloride was added dropwise and with mechanical stirring. The rate of addition was regulated so that the temperature of the reaction mixture did not rise above 50°. The addition, which required 1.5 hours, was followed by one-half hour of stirring. The hydrogen chloride was removed as much as possible under reduced pressure at room temperature. The reaction mixture was surrounded by a water-bath at 5°, and a few grams of calcium carbonate was added with thorough stirring. Then, as the mixture became thicker, a suspension of calcium carbonate in water was added until the color turned bright yellow, carbon dioxide was no longer evolved, and the

(7) We are indebted to Dr. Charles Rosenblum and his associates for the ultraviolet absorption measurements.

(8) Stiller, Keresztesy and Stevens, THIS JOURNAL, **61**, 1237 (1939).

(9) Paper IV of this series.

(10) Scudi, *J. Biol. Chem.*, **139**, 707 (1941).

(11) Karrer and Viscontini, *Helv. Chim. Acta*, **30**, 52 (1947).

(12) Gale, *Adv. in Enzymology*, **6**, 28 (1946).

(13) Harris, Webb and Folkers, THIS JOURNAL, **62**, 3198 (1940).

(14) We are indebted to Mr. Richard Boos and his associates for the microanalyses.

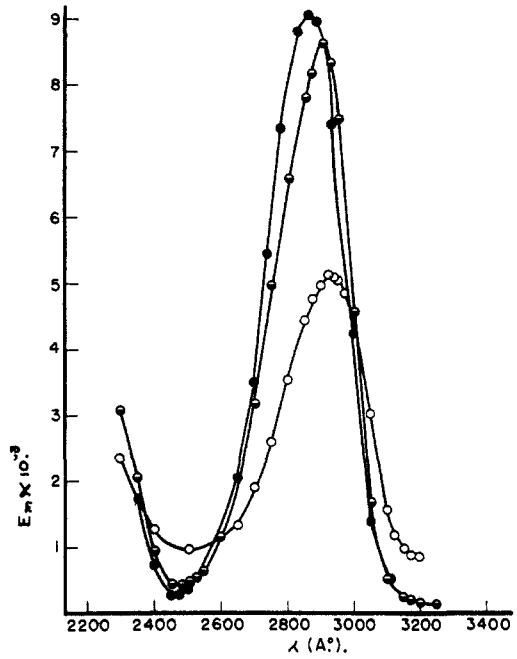
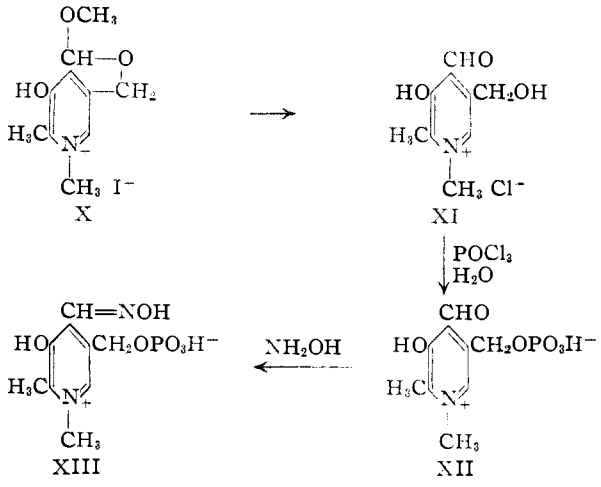


Fig. 2.—Absorption spectra at *p*H 1.9: ●, pyridoxal; O, codecarboxylase; ⊖, pyridoxal monoethylacetal.



solution had reached a *p*H of 5. After the mixture had been chilled in an ice-bath for one hour, the precipitate was removed by filtration and washed three times with ice-water; the volume of the clear yellow filtrate plus the washings totaled 110 ml. This solution was diluted with three times its volume of ethyl alcohol and chilled for two hours. The yellow precipitate was centrifuged and was washed twice with alcohol and once with ether. After drying, the monocalcium salt of codecarboxylase weighed 0.46 g. (7%). By biological assay[4] the material was estimated to be about 80% calcium codecarboxylase.

Pyridoxal could be reclaimed from the filtrate in the following manner: The filtrate was concentrated and treated with hydroxylamine hydrochloride and sodium bicarbonate (solution adjusted to *p*H 7.5). The pyridoxal oxime was collected on a filter and washed in turn with water, alcohol and ether. The yield of recovered oxime was 1.8 g.; m.p. 224–224.5°. The melting point of an authentic specimen of pyridoxal oxime showed no depression when the two oximes were mixed.

Codecarboxylase Oxime.—A sample of the calcium salt of codecarboxylase weighing 254 mg. (estimated by assay to be about 80% pure) was suspended in 10 ml. of water. Hydroxylamine hydrochloride (250 mg.) was added, followed by five drops of 6 *N* hydrochloric acid. The yellow color disappeared, and the white codecarboxylase oxime crystallized at once from the acid solution. After cooling in an ice-bath, the oxime was filtered and washed successively with water, alcohol and ether. It melted at 229–230° (dec.) and weighed 156 mg.

Anal. Calcd. for $C_8H_{11}N_2O_6P$: C, 36.64; H, 4.23; N, 10.69; P, 11.83. Found: C, 36.45; H, 4.49; N, 10.41; P, 11.89.

Codecarboxylase Oxime (Phosphorylation of Pyridoxal Oxime).—To a suspension of 5.00 g. of pyridoxal oxime in 35 ml. of water, 12 ml. of phosphorus oxychloride was added dropwise and with stirring. The temperature of the reaction mixture was never allowed to exceed 35°. After an additional half-hour stirring, the hydrochloride of unreacted pyridoxal oxime was removed by collection on a filter. The filtrate was cooled to 5–10°, and kept at that temperature while a suspension of 75 g. of calcium carbonate in 50 ml. of water was added. The *p*H of the resulting mixture was about 4.5. After an additional hour chilling, the precipitated calcium phosphate was collected on a filter and washed with ice-water. The filtrate was clarified by filtration through a fine sintered glass disc. The 100 ml. of solution was diluted with 300 ml. of ethyl alcohol, and the small amount of material which precipitated on chilling was removed by centrifuging. Ammonium hydroxide was added to the solution until the *p*H was 8, and the mixture was stored at 5° overnight. The resulting crude calcium salt of the phosphate of pyridoxal oxime was separated by centrifuging, washed twice with ethyl alcohol and once with ether, and dried; yield 0.57 g. This crude calcium salt was ground to a fine powder and suspended in about 10 ml. of ice-water. Concentrated hydrochloric acid was added until the mixture showed a *p*H of 2. The crystals of the phosphate of pyridoxal oxime were collected on a filter, ground thoroughly with water, again collected on a filter, and washed thoroughly with water, alcohol and ether; yield 0.15 g. (2%); m.p. 220–222° (dec.). A mixture of this material and the oxime prepared from codecarboxylase showed no depression in melting point. The discrepancy between the melting points for this preparation and the one described above is due to variations in the rate of decomposition.

Anal. Calcd. for $C_8H_{11}N_2O_6P$: C, 36.64; H, 4.23; N, 10.69; P, 11.83. Found: C, 36.40; H, 4.51; N, 10.52; P, 12.1.

Calcium Salt of 2-Methyl-3-hydroxy-4-hydroxymethyl-5-pyridylmethylphosphoric Acid (II).—To a solution of 5.0 g. of pyridoxine hydrochloride in 35 ml. of water was added dropwise, and with stirring, 25 ml. of phosphorus oxychloride. The temperature was kept at 50° until the addition was complete, and then it was quickly raised to 100° for a brief period. After one-half hour of stirring, excess hydrogen chloride was removed under diminished pressure at room temperature, the mixture was surrounded by a water-bath at 5°, and a thick suspension of calcium carbonate in water was added until carbon dioxide ceased to be evolved and the *p*H of the solution was 3. After an hour of chilling in an ice-bath, the mixture was filtered and the precipitate washed three times with ice-water. The combined filtrate and washings (95 ml.) were diluted with three volumes of alcohol, cooled in ice for two hours, and centrifuged. The precipitate was washed twice with alcohol and once with ether. The yield of crude calcium salt of 2-methyl-3-hydroxy-4-hydroxymethyl-5-pyridylmethylphosphoric acid was 0.79 g. Another 0.57 g. of material was obtained when the filtrate was adjusted to *p*H 8 with ammonium hydroxide, then chilled overnight, centrifuged and the precipitate washed as before.

Codecarboxylase Oxime (Oxidation of 2-Methyl-3-hydroxy-4-hydroxymethyl-5-pyridylmethylphosphoric Acid).—To a suspension of 0.93 g. of the calcium salt of 2-methyl-3-hydroxy-4-hydroxymethyl-5-pyridylmethylphosphoric acid (II) and 0.37 g. of manganese dioxide in 11 ml. of water, 1.8 ml. of 6 *N* sulfuric acid was added dropwise. The reaction mixture was heated to 70° with stirring, and held at that temperature for 20 minutes. After collection of the solid material on a filter, 0.24 g. of hydroxylamine hydrochloride was added with stirring. The solution was warmed briefly on the steam-bath, then chilled, and the crystals of the codecarboxylase oxime were collected on a filter and washed well with water, alcohol and ether; yield 60 mg. After one recrystallization from water, the melting point was 227–228° (dec.), and the melting point of a mixture of this

material with an authentic specimen of codecarboxylase oxime showed no depression.

Anal. Calcd. for $C_8H_{11}N_2O_6P$: C, 36.64; H, 4.23; N, 10.69. Found: C, 36.65; H, 4.31; N, 10.78.

Pyridoxal O-Methyloxime (VII).—A solution of 2.0 g. of pyridoxal hydrochloride and 1.5 g. of methoxylamine hydrochloride in a little water was adjusted to *p*H 8 with sodium acetate. After heating on the steam-bath for a few minutes, the solution was cooled in an ice-bath. Pyridoxal O-methyloxime was collected on a filter, washed thoroughly with water, and dried in a vacuum oven; yield 1.8 g. (90%). After a recrystallization from alcohol–water and another from alcohol, the O-methyloxime melted at 159–160°.

Anal. Calcd. for $C_9H_{12}N_2O_3$: C, 55.09; H, 6.17; N, 14.28. Found: C, 55.15; H, 6.24; N, 14.10.

O-Methyloxime of Codecarboxylase (VIII).—A suspension of 0.32 g. of crude calcium codecarboxylase preparation in a little ice-water was treated dropwise with 6 *N* hydrochloric acid until the material was essentially in solution. A little amorphous material was removed by filtering. After the addition of 100 mg. of methoxylamine hydrochloride, crystals developed slowly. The O-methyloxime of codecarboxylase, which was collected on a filter and washed with water, alcohol and ether, melted at 212–213° (dec.). It was dried at 100° (1 mm.).

Anal. Calcd. for $C_9H_{13}N_2O_6P$: C, 39.13; H, 4.74; N, 10.14; P, 11.23. Found: C, 39.16; H, 5.01; N, 9.92; P, 11.18.

O-Methyloxime of 2-Methyl-3-acetoxy-4-formyl-5-hydroxymethylpyridine (IX).—To a suspension of 3.6 g. of pyridoxal O-methyloxime (VII) in 50 ml. of ice-water, 5 ml. of acetic anhydride was added. The mixture was well shaken, and the lumps broken. After the addition of another 5 ml. of acetic anhydride, thorough mixing, and cooling, the resulting crystals were collected on a filter, washed well with water, and dried in a vacuum oven. O-Methyloxime of 2-methyl-3-acetoxy-4-formyl-5-hydroxymethylpyridine was obtained in a yield of 3.1 g. (71%); m.p. 137–138°. Recrystallization of the compound from methyl alcohol–water did not change the melting point.

Anal. Calcd. for $C_{11}H_{14}N_2O_4$: C, 55.45; H, 5.92; N, 11.76. Found: C, 54.96; H, 5.73; N, 11.79.

Pyridoxal Monomethylacetal (1-Ethoxy-1,3-dihydro-6-methylfuro[3,4-c]pyridin-7-ol).—Eight grams of pyridoxal hydrochloride was suspended in 50 ml. of methyl alcohol and the solution was refluxed for 15 minutes. After cooling, 3.3 g. of sodium bicarbonate was added, and the mixture was refluxed for an additional hour and a half. The precipitated sodium chloride was separated from the cooled solution by filtration and washed well with methyl alcohol. The combined filtrate and washings were chilled. The small amount of pyridoxal monomethylacetal which precipitated was washed well with methyl alcohol and ether. It was dried at 100° (1 mm.); m.p. 169–170°. The material remaining in solution was used directly, without isolation.

Anal. Calcd. for $C_9H_{11}NO_3$: C, 59.66; H, 6.12; N, 7.73. Found: C, 59.63; H, 6.09; N, 7.77.

Monomethyl Acetal of Pyridoxal Methiodide (X).—One gram of pyridoxal hydrochloride was converted to the monomethyl acetal. Two hundred milliliters of benzene, added to the methyl alcohol solution, caused a turbidity which was removed by filtering, and after 15 ml. of methyl iodide had been added, the solution was refluxed for 16 hours. The solution was distilled to dryness in a warm water-bath, under reduced pressure. The yellow residue crystallized from methanol–ether to yield 0.98 g. (60%) of the monomethyl acetal of pyridoxal methiodide, which after one recrystallization dec. 178–179°.

Anal. Calcd. for $C_{10}H_{14}NO_3I$: C, 37.17; H, 4.37; N, 4.34. Found: C, 37.29; H, 4.20; N, 4.13.

Pyridoxal Methochloride (XI).—A solution of 4.6 g. of the monomethyl acetal of pyridoxal methiodide in 70 ml. of water was shaken with 2.5 g. of silver chloride for two hours. After collection of the solid material on a filter, the filtrate no longer contained iodide ion. After acidification to *p*H 2 with hydrochloric acid, the solution was evaporated to dryness under reduced pressure, and the residue crystallized twice from water–acetone. The crystals of pyridoxal methochloride, weighing 1.1 g. (35%), melted in part around 100°, resolidified, and decomposed gradually above 160°.

Anal. Calcd. for $C_9H_{12}NO_3Cl$: C, 49.66; H, 5.56; N, 6.44; N-CH_3, 6.93. Found: C, 50.16; H, 5.49; N, 6.8; N-CH_3, 5.5.

Calcium Salt of the Betaine of 1,2-Dimethyl-3-hydroxy-4-formyl-5-pyridylmethylphosphoric Acid (XII).—A mixture of 3.58 g. of the monomethyl acetal of pyridoxal methiodide (X) and 1.95 g. of silver chloride suspended in 30 ml. of water was shaken mechanically for two hours. The solid material was collected on a filter, and the filtrate was found to be free of iodide ion. The filtrate was concentrated to 25 ml., surrounded by a cold water-bath, and treated dropwise with 18 ml. of phosphorus oxychloride. The mixture was stirred mechanically, and the temperature was maintained at 40–45°. The addition, which required an hour, was followed by 45 minutes of stirring. After excess hydrogen chloride had been removed under reduced pressure, the reaction mixture was surrounded by a bath at 5–10°, and 150 g. of calcium carbonate dispersed in 120 ml. of water was added with mechanical stirring. The mixture was chilled for an hour, and the precipitate was collected on a filter. The precipitate was washed well with ice-water. The combined filtrate and washings were clarified by filtering through a sintered glass disc. The 110 ml. of filtrate was diluted with 330 ml. of alcohol, and chilled. The crude calcium salt of the betaine of 1,2-dimethyl-3-hydroxy-4-formyl-5-pyridylmethylphosphoric acid, obtained in a yield of 0.15 g., was centrifuged and washed twice with alcohol and once with ether.

Oxime of the Betaine of 1,2-Dimethyl-3-hydroxy-4-formyl-5-pyridylmethylphosphoric Acid (XIII).—A suspension of 0.15 g. of the calcium salt of the betaine of 1,2-dimethyl-3-hydroxy-4-formyl-5-pyridylmethylphosphoric acid in a little ice-water was treated with six drops of 6 *N* hydrochloric acid. The insoluble material was separated by centrifuging, and washed twice with ice-water. The combined filtrate and washings were filtered through a sintered glass disc. The clear solution, after the addition of 0.04 g. of hydroxylamine hydrochloride, was warmed to room temperature. After thorough chilling, the crystals of the oxime of the betaine of 1,2-dimethyl-3-hydroxy-4-formyl-5-pyridylmethylphosphoric acid were collected on a filter and washed well, in succession, with ice-water, alcohol and ether; yield 0.02 g. This preparation, m.p. 224–225° (dec.), together with another similar one was ground and dried at 100° (1 mm.) for analysis. The material was hygroscopic.

Anal. Calcd. for $C_9H_{13}N_2O_6P$: C, 39.14; H, 4.74; N, 10.15; P, 11.19. Found: C, 38.70; H, 5.12; N, 10.14; P, 10.97.

RAHWAY, N. J. RECEIVED JANUARY 12, 1951