cation of the filtrate. The precipitate was recrystallized from petroleum ether to give colorless needles (m. p. 114°).

*Anal.* Calcd. for $C_6HOBr_4F$: Br, 74.73. Found: Br, 74.47.

**The Reduction of 3-Fluoro-2,4,6-tribromophenol Bromide.**—This bromide was reduced with zinc and hydrochloric acid. The product was isolated by steam distillation after dilution with some water. Upon crystallization from petroleum ether, the product melted at 94° and showed no depression with a sample of 3-fluoro-2,4,6-tribromophenol.

*Anal.* Calcd. for $C_6H_2OBr_3F$: Br, 68.75. Found: Br, 68.82.

**Behavior of 3-Fluoro-2,4,6-tribromophenol Bromide toward Phenylhydrazine.**—Some 3-fluoro-2,4,6-tribromophenol bromide was dissolved in dioxane and poured into a solution containing an excess of phenylhydrazine in a mixture of dioxane and glacial acetic acid. After purification by sublimation the product melted at 94° and showed no depression with 3-fluoro-2,4,6-tribromophenol.

**Action of Silver Fluoride on 3-Fluoro-2,4,6-tribromophenol Bromide.**—Ten grams of this compound was mixed with 10 g. of solid silver fluoride, suspended in 250 ml. of acetone, and stirred for several hours. The silver bromide was filtered off and the filtrate was diluted with water to a volume of 1200 ml. A small amount (less than a gram) of bright yellow platelets, m. p. 169° precipitated and was filtered off.

*Anal.* Calcd. for $(C_6HOB_3F)_2$: F, 5.46. Found: F, 5.5.

**Action of Lead Acetate on 3-Fluoro-2,4,6-tribromophenol Bromide.**—A mixture of 4.3 g. of the bromide and 15 g. of lead acetate trihydrate in glacial acetic acid was stirred and kept at 70° for four hours and then allowed to stand for twelve hours. The solution was decanted from the lead bromide, diluted with water and extracted with ether. The ether solution was washed with very dilute sodium hydroxide to remove acetic acid, then with water, and finally was dried over calcium chloride. Most of the ether was distilled off and the rest removed under vacuum. The viscous residue was dissolved in ether then precipitated with petroleum ether as a white granular solid, almost insoluble in all of the common solvents. Upon heating it became slightly pink at about 140° and melted to a red oil at 162° with decomposition. It liberated iodine from potassium iodide in the cold.

*Anal.* Calcd. for $(C_6HOBr_3F)_2$: Br, 68.97. Found: Br, 69.27.

## Summary

The preparation of 2,6-dibromo-3-fluorophenol, 2,4,6-tribromo-3-fluorophenol and 2,6-dibromo-4-fluorophenol had been repeated. The following new compounds have been obtained: 2-fluoro-4,6-dibromophenol, 2,4,5,6-tetrabromo-3-fluorophenol, and 2,4,6-tribromo-3-fluorophenol bromide. The bromination of the three fluorophenols with an excess of bromine has been studied and an attempt has been made to elucidate the structure of 3-fluorotribromophenol bromide.

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[CONTRIBUTION FROM THE UNIVERSITY OF TEXAS, BIOCHEMICAL INSTITUTE, AND THE CLAYTON FOUNDATION FOR RESEARCH]

# The Vitamin $B_6$ Group. I. Formation of Additional Members from Pyridoxine and Evidence Concerning their Structure

BY ESMOND E. SNELL

Evidence for the occurrence in natural materials of one or more substances tentatively called "pseudopyridoxine" has been presented in detail.[1] The existence of such a substance was discovered because on suitable pyridoxine-free media, natural extracts were much more active in promoting growth of various lactic acid bacteria than could be explained by their pyridoxine content, as determined by other methods. Behavior on fractionation indicated that the substance was chemically similar to pyridoxine. Metabolic experiments with rats and human beings demonstrated that pyridoxine was partially converted into "pseudopyridoxine" by the animal organism. Experiments with *Streptococcus lactis* R[2] (*Streptococcus fecalis* R) showed that almost no pyridoxine was absorbed from media in which the concentration of this substance appeared to limit growth. It was assumed that the organism transformed only a minute amount of the pyridoxine present to the more active "pseudopyridoxine," and that the latter was the substance absorbed and utilized for growth purposes.

It was later shown[3] that the activity of pyridoxine for this organism was greatly increased by autoclaving with the basal medium used for assay. This increased activity was traced to interaction of pyridoxine with the amino acids of the medium. This explained why growth of *S. fecalis* R ceased even though pyridoxine was still present; the substance promoting growth was the product formed by autoclaving pyridoxine with amino acids and this was formed in very low yield by the procedure used. This production from pyridoxine by purely chemical means of a substance with heightened growth-promoting activity suggested that other more effective procedures for producing the transformation might be found, and that this approach to the problem might be more economical than direct isolation of the active substance from natural materials.

The activity of two synthetic compounds, pyridoxamine and pyridoxal, structures for which were suggested by this approach, has been briefly described.[4] The present paper describes work

(1) Snell, Guirard and Williams, *J. Biol. Chem.*, **143**, 519 (1942).

(2) Niven and Sherman (*J. Bact.*, **47**, 335 (1944)) have shown that this organism is actually an enterococcus, physiologically and serologically identical with *Streptococcus fecalis*. The older designation is used here for purposes of continuity. Henceforward, this organism will be designated as *Streptococcus fecalis* R.

(3) Snell, *Proc. Soc. Exptl. Biol. Med.*, **51**, 356 (1942).

(4) Snell, *J. Biol. Chem.*, **154**, 313 (1944).

which implicated these structures as possible active compounds.

## Experimental

**Testing Procedures.**—The basal medium and conditions previously used[1] were employed in the early part of the present work. Later, the medium was modified by inclusion of 1 mg. of asparagine and 1 $\gamma$ of *p*-aminobenzoic acid per 10 cc. This modified medium permitted good growth of both *Lactobacillus casei* which was used for many assays, and *S. fecalis* R which was used in the majority of tests. Occasionally, with the latter organism, the test failed because profuse growth occurred in the control tubes without added pyridoxine. This situation was remedied by repeating the charcoal treatment of the casein hydrolysate,[1] and by addition of 1 mg. of glycine per tube. It has been shown[5] that glycine acts as a growth depressant for *S. fecalis*, the effect of which is specifically counteracted by pyridoxine or substances which can replace pyridoxine for this organism. For routine assays, it was found most convenient to compare activities of fractions as determined in media which had been sterilized by autoclaving at 15 lb. steam pressure for ten minutes,[1] despite the fact that such treatment increases the activity of the pyridoxine standard by several fold for *S. fecalis*.[3] Because the extent of this increased activity is somewhat variable, and because of other properties of the active compounds, tests made in this manner from day to day are comparable in a qualitative manner only. When quantitative comparisons were desired, all samples were run in a single test. A few pyridoxine determinations were made with yeast by the method of Williams, *et al.*[6]

**Effect of Miscellaneous Chemical Treatments on Activity of Pyridoxine for *S. fecalis* R.**—The effects of a number of miscellaneous treatments of pyridoxine on its activity for *S. fecalis* are shown in Table I. In each case, the reaction

TABLE I

EFFECT OF MISCELLANEOUS TREATMENTS ON ACTIVITY OF PYRIDOXINE FOR *S. fecalis* R

| Experiment | Treatment | Activity of product (pyridoxine = 1.0) |
|---|---|---|
| 1 | Heated with NaOH | 2.5 |
| 2 | Heated with HCl | 1.2 |
| 3 | Heated with $NH_3$ | 22.0 |
| 4 | Heated with sodium acetate | 1.1 |
| 5 | Heated with cystine and sodium acetate[a] | 10.6 |
| 6 | Acetylation with acetic anhydride in pyridine | 20 |
| 7 | Acetylation with acetyl chloride | 13 |
| 8 | Acetylated, then hydrolyzed with NaOH | 1.0 |
| 9 | Oxidation with potassium permanganate | 160 |

[a] The effect of autoclaving with a variety of amino acids has been presented previously.[3]

mixture resulting from the treatments described below was neutralized where necessary, properly diluted, and assayed. Figures in the table represent the increase in activity, if any, over that of pyridoxine hydrochloride for this test organism:

1. Heating with sodium hydroxide: 3.1 mg. of pyridoxine hydrochloride was dissolved in 0.31 cc. of 1 *N* sodium hydroxide. The solution was autoclaved at 15 lb. pressure for four hours.
2. Heating with hydrochloric acid: 1 mg. of pyridoxine hydrochloride dissolved in 0.2 cc. of 18% hydrochloric acid was autoclaved as above for four hours.
3. Heating with ammonia: To 6.7 mg. of pyridoxine in a Pyrex test-tube was added 1 cc. of a 20% solution of ammonia in methanol. The tube was sealed in an oxygen flame, heated at 100° for two hours, cooled and opened.

4–5. Heating with sodium acetate with and without cystine: To 10 cc. of solution containing 10 mg. of cystine and 50 mg. of sodium acetate was added 0.1 mg. of pyridoxine hydrochloride in 0.1 cc. of water. The mixture was autoclaved at 15 lb. steam pressure for thirty minutes. A control was carried in the same manner without added cystine.

6. Acetylation with acetic anhydride in pyridine: To 10 cc. of acetic anhydride containing 2 cc. of dried pyridine was added 14 mg. of pyridoxine hydrochloride. The mixture was refluxed for one hour, then concentrated to dryness under reduced pressure. The residue was dissolved in methanol, and an aliquot diluted with water for testing.
7. Acetylation with acetyl chloride: To 10 cc. of acetyl chloride was added 5 mg. of pyridoxine hydrochloride. The mixture was refluxed for one-half hour, concentrated to dryness under reduced pressure, the residue dissolved in methanol, and an aliquot diluted with water for testing.
8. Hydrolysis of acetylated mixture with sodium hydroxide: To 1 cc. of the methanol solution obtained in 6, equivalent to 1.0 mg. of original pyridoxine hydrochloride, was added 1 cc. of aqueous 2 *N* sodium hydroxide. The mixture was heated at 100° for two hours.
9. Oxidation with potassium permanganate: To 1 cc. of a solution containing 1.0 mg. of pyridoxine hydrochloride was added 1 cc. of a solution containing 1.0 mg. of potassium permanganate. A color change began almost immediately with formation of brown manganese dioxide. After fifteen minutes at room temperature, the solution was diluted for assay.

Results show that several treatments of pyridoxine result in considerable increase in activity for *S. fecalis*. Of these, oxidation was most effective, followed by ammonia treatment, acetylation, and heating with cystine. The effectiveness of such diverse procedures in producing activity increases would indicate that more than one product had such increased activity for this organism.

**Activity of Pyridoxine Analogs for Lactic Acid Bacteria and Yeast.**—To make certain that the products with increased activity obtained above were not known compounds, a number of synthetic compounds related to pyridoxine in structure were tested for activity. These were supplied through the courtesy of Dr. Karl Folkers. Results are given in Table II. Only the diacetate of pyridoxine, compound 8046, showed greater activity than pyridoxine for any of the test organisms. This heightened activity of the diacetate is in accord with that of the acetylated products (presumably pyridoxine triacetate) prepared from pyridoxine and listed in Table I. For other organisms[7,8,9] all analogs of pyridoxine tested possessed much lower growth-promoting activity than did pyridoxine, with the exception of compounds like the di- and tri-acetates of pyridoxine, which could be hydrolyzed by the test organisms to yield pyridoxine.

The heightened activity of pyridoxine esters can be interpreted in various ways: (a) the increased activity resides in the pure acetylated product; (b) the increased activity is a property of a by-product formed in the acetylation reaction; or (c) pyridoxine esters, like pyridoxine, are increased in activity when heated with the medium during sterilization, and the reaction forming the product with heightened activity takes place to a greater extent with esters of pyridoxine than with the unacetylated product.

(5) Snell and Guirard, *Proc. Natl. Acad. Sci.*, **29**, 66 (1943).

(6) Williams, Eakin and McMahan, *Univ. Tex. Publ.*, **4137**, 24 (1941).

(7) Unna, *Proc. Soc. Expt. Biol. Med.*, **43**, 122 (1940).

(8) Bohonos, Hutchings and Peterson, *J. Bact.*, **44**, 479 (1942).

(9) Robbins and Ma, *Bull. Torrey Bot. Club*, [5] **69**, 342 (1942).

TABLE II

ACTIVITY OF CERTAIN PYRIDOXINE ANALOGS FOR LACTIC ACID BACTERIA AND YEAST

| Compound number | Compound | Potency | | |
|---|---|---|---|---|
| | | *S. fecalis* R | *L. casei* | *S. cerevisiae* |
| | Pyridoxine hydrochloride (2-methyl-3-hydroxy-4,5-bis-(hydroxymethyl)-pyridine hydrochloride) | 1.0 | 1.0 | 1.0 |
| 7619 | Pyridoxine-N-methylbetaine | 0.001 | 0.001 | 0.0038 |
| 8043 | 2,4-Dimethyl-3-hydroxy-5-hydroxymethylpyridine hydrochloride | 0.058 | 0.082 | 0.055 |
| 8044 | 2-Methyl-3-hydroxy-4-methoxymethyl-5-hydroxymethylpyridine hydrochloride | 0.58 | 0.51 | 0.11 |
| 8045 | 2-Methyl-3-amino-4-methoxymethyl-5-aminomethylpyridine dihydrochloride | 0.001 | 0.001 | 0.003 |
| 8046 | 2-Methyl-3-hydroxy-4,5-bisacetoxymethylpyridine hydrochloride | 7.0 | 4.7 | 1.1 |
| 8047 | 2-Methyl-3-hydroxy-4,5-epoxydimethylpyridine hydrochloride | 0.15 | 0.61 | 0.043 |

Reduction of the activity of acetylated pyridoxine to that of the unacetylated product by alkaline hydrolysis (Table I) favors (a) or (c) as an explanation; (b) is further discounted by the activity of the purified pyridoxine diacetate (Table II). Experimental test of (c) revealed that the activity of pyridoxine acetates in promoting growth of *S. fecalis* was reduced when the compounds were tested without autoclaving with the medium, and became progressively greater the longer they were autoclaved with the medium. Thus (c) appears to be the correct explanation for the increased activity of the acetates.

**Effect of Ammonia Treatment of Pyridoxine Esters.**—The fact that acetylated pyridoxine is converted even more readily to active products by heating with the basal medium than is pyridoxine suggests application to the acetates of other procedures which increased activity of pyridoxine (Table I).

Details of some such experiments are given below. The activity of all products obtained was compared with that of pyridoxine hydrochloride which is ascribed a potency equal to 1.0.

**Experiment 1.** a. Pyridoxine hydrochloride was acetylated with acetic anhydride in pyridine, essentially as described for Expt. 6, Table I. The crude dried oil obtained was dissolved in methanol, and a portion diluted for assay: Potency = 10.0.

b. To 1 cc. of a methanol solution containing 1.0 mg. of the crude acetate of pyridoxine was added 1 cc. of 5 *N* sodium hydroxide. The mixture was heated in a steam cone for fifteen minutes: Potency = 1.0.

c. A mixture prepared as in (b) was heated for one and one-half hours, then assayed: Potency = 1.5 (*cf.* Table I, Expt. 1).

d. To 1 cc. of methanol solution containing 1.0 mg. of the crude acetate of pyridoxine was added 1 cc. of a 20% solution of ammonia in methanol. The tube was sealed and heated at 100° for one hour, cooled, and opened: Potency of product = 93.

e. A separate tube, treated just as in (d) was opened, and 1 cc. of 5 *N* sodium hydroxide added. After heating at 100° for thirty minutes, the product was assayed: Potency = 155.

The heightened activity observed when acetylated pyridoxine is assayed is not evident after hydrolysis with sodium hydroxide. Prolonged heating of the hydrolyzed product with sodium hydroxide results in slightly increased activity, as previously noted. When the product is heated with ammonia, a very great increase in activity is observed. The active product formed is stable to hydrolysis with sodium hydroxide; in fact, a further increase in activity appears to be produced by hydrolysis. As appears below, this latter increase is likely produced by complete deacetylation of the active product by sodium hydroxide, a change only incompletely effected by the ammonia treatment (*cf.* Expts. 3d and e).

**Experiment 2.**—To determine whether the pure 4,5-diacetoxypyridoxine (Merck) would undergo the same changes as the crude acetylated pyridoxine, portions of this product were treated as in (d) above: Potency of the diacetate = 7.0; Potency after ammonia treatment = 110.0.

**Experiment 3.**—a. 2 g. of pyridoxine hydrochloride was benzoylated in alkaline solution with benzoyl chloride. The product was recrystallized from methanol. It melted at 120–121°, and contained 2.94% N (Dumas). Ichiba and Michi[10] give the melting point of pyridoxine tribenzoate as 121–122°; its theoretical nitrogen content is 2.91%. A portion dissolved and diluted for assay showed a potency of 3.6.

b. To 97.5 mg. of pyridoxine tribenzoate was added 2 cc. of a 20% solution of ammonia in methanol. The mixture was heated in a sealed tube at 100° for one hour, cooled and opened. A portion diluted for assay showed a potency of 52.

c. A 2.6-cc. portion of methanol containing reaction product produced in (b) above equivalent to 26 mg. of original pyridoxine hydrochloride was added to 2.6 cc. of 5 *N* sodium hydroxide and heated at 100° for twenty minutes. 0.2 cc. was diluted for testing: Potency = 300.

d. To the remaining 5.0 cc. of hydrolyzate in (c) there was added 0.5 cc. of benzoyl chloride; the mixture was shaken, and the benzoylated products extracted with ether, the ether solution dried, and a portion diluted for assay: Potency = 22.

e. A portion of the rebenzoylated material obtained in (d) above was hydrolyzed by heating thirty minutes at 100° with 2.5 *N* sodium hydroxide: Potency = 300.

From the above experiments, it can be concluded that triesters of pyridoxine, and di-esters in which the 4,5-*bis*-(hydroxymethyl) groups are esterified, react in the presence of alcoholic ammonia to giving a product with greatly increased activity for *S. fecalis*. The product thus produced is stable to alkali. Esterification of it greatly reduces its activity; this is regenerated by again hydrolyzing with alkali.

A large number of experiments were run in an attempt to determine conditions which would permit a maximum yield of active product to be formed. It seems of little value to present these in detail. With methanol as solvent, and with constant amounts of pyridoxine tribenzoate, the activity of the product obtained increases with increasing concentration of ammonia. A maximum is reached, however, so that even liquid ammonia does not give higher yields than that recorded in Expt. 3c above. The solvent can be varied markedly without greatly influencing the result. Activation proceeds about equally well in methanol, ethanol, *n*-propanol, *n*-butanol and ethyl acetate. Mixtures of methanol and water are also effective. The principal requisite for success appears to be a solvent in which the pyridoxine ester and ammonia are mutually soluble. Concentrated ammonium hydroxide solutions may be substituted for solutions of ammonia in methanol with only slight and irregular decreases in activity of the product obtained. With methanol as solvent, and the con-

(10) Ichiba and Michi, *Sci. Papers Inst. Phys. Chem. Res.* (Tokyo), **35**, 73 (1939).

centration of ammonia kept constant, the activity of the product obtained decreases slightly as the concentration of pyridoxine tribenzoate is increased. Prolonged heating times do not markedly increase activity of products obtained over that already recorded.

Of greater interest is the specificity of ammonia in producing the activations. For experiments recorded in Table III, either pyridoxine tribenzoate or crude acetylated pyridoxine (pyridoxine triacetate) was heated in methanol–water solution containing about 10% of the reagent the effect of which was to be determined. Only ammonia, or substances like ammonium carbonate which yield ammonia on heating, were effective in producing the activation. It has already been pointed out, however, that a similar but a less marked increase in activity of the esters occurs on heating very dilute aqueous solutions of them with the basal medium used for assay.

TABLE III

SPECIFICITY OF AMMONIA IN PRODUCTION OF A SUBSTANCE WITH INCREASED GROWTH-PROMOTING ACTIVITY FOR *S. fecalis* R.

| Pyridoxine ester[a] heated with | Activity of product (pyridoxine hydrochloride = 1.0) |
|---|---|
| Ammonia | 80–400 |
| Pyridine | 1.0 |
| Piperidine | 1.0 |
| Methylamine | 1.0 |
| Trimethylamine | 1.0 |
| Diethanolamine | 1.0 |
| Sodium carbonate | 1.0 |
| Sodium bicarbonate | 1.0 |
| Ammonium carbonate | 200 |

[a] All reaction mixtures were hydrolyzed with 2.5 *N* sodium hydroxide before testing.

This specificity of ammonia suggests that it actually participates in the reaction by which products of increased activity are formed. Inspection of the formula for pyridoxine would lead one to suspect that the only positions open to attack by ammonia are the three bearing hydroxyl groups, with consequent replacement of one or more of these by amino groups. If the product formed is an amine, it should be destroyed by nitrous acid. This was tested as follows.

Pyridoxine tribenzoate was heated with ammonia in methanol, and the activated product hydrolyzed with sodium hydroxide as described above. To 0.2 cc. of the neutralized hydrolyzate (equivalent to 2 mg. of pyridoxine hydrochloride) was added 0.1 cc. glacial acetic acid and 1 cc. of a 1% solution of sodium nitrite. Two milligrams of pyridoxine hydrochloride was treated with glacial acetic acid and sodium nitrite in exactly the same manner as a control. Both solutions were shaken for one and one-half hours; then 10 mg. of glycine was added to destroy excess nitrous acid, the solutions allowed to stand for two hours, then assayed with *S. fecalis*. Results were

| | Potency |
|---|---|
| Pyridoxine hydrochloride | 1.0 |
| Pyridoxine hydrochloride, nitrous acid-treated | 1.1 |
| "Ammonia activated" pyridoxine | 105 |
| "Ammonia activated" pyridoxine, nitrous acid-treated | 2.5 |

Pyridoxine was unaffected by nitrous acid treatment[11]; the activity of "ammonia activated" pyridoxine, on the other hand, was 98% destroyed by nitrous acid treatment.

Destruction of the active product by nitrous acid, together with the specificity of ammonia in producing the substance with increased activity from esters of pyridoxine, was taken as conclusive evidence that the active substance produced was an amine. Seven possible amines, derived from pyridoxine by replacement of one or more hydroxyl groups by amino groups thus become suspect.

**Partial Purification of the Amine and its Activity for Other Organisms.**—With the synthetic product now available,[4,12] detailed description of attempts at purification of the active material are superfluous. The most successful method tried involved heating pyridoxine tribenzoate (0.5 g.) with alcoholic ammonia, hydrolysis of the resultant solution with sodium hydroxide, and reacylation of the "activated" mixture with azobenzene-4-carboxylic acid chloride (azoyl chloride[13]). The deep-red material resulting was dissolved in chloroform and fractionated on an aluminum oxide column. Elution of esters of the active material was effected by washing the column with chloroform–ethanol (4:1); a large amount of inactive materials was left on the column. Repetition of the fractionation, using only the active fraction obtained above, yielded 110 mg. of an active azoate. A portion of this was hydrolyzed and assayed

| | Activity (pyridoxine hydrochloride = 1.0) |
|---|---|
| *S. fecalis* | 490 |
| *L. casei* | 1.5 |
| Yeast | 0.20 |

A triazoyl ester of pyridoxine should contain 25.9% pyridoxine, expressed as the hydrochloride; the above product showed activity with yeast equivalent to a content of 20% pyridoxine hydrochloride. If it is assumed that the active amine has about the same activity for yeast as does pyridoxine,[cf. 4] the above material, if unesterified, would be 2500 times as active as an equal weight of pyridoxine hydrochloride in promoting growth of *S. fecalis*, but only 7.5 times as active for *L. casei*. These figures compare rather well with the activity of pure pyridoxamine for these organisms, as later determined,[4,14] and indicate that the above ester was almost pure. Due to subsequent developments, the product was not further investigated.

**Effect of Partial Oxidation of Pyridoxine on its Growth-Promoting Activity.**—The effect of potassium permanganate oxidation on growth-promoting activity of pyridoxine was noted in Table I. The product formed could scarcely be an amine, and was therefore separately investigated.

The effect of a variety of oxidizing agents in producing this increased activity of pyridoxine is summarized in Table IV. Of the oxidizing agents tried, only permanganates, chromates and dichromates, and manganese dioxide were effective in producing the change; potassium chlorate was very slightly effective. Bleaching powder, Fehling solution, alkaline iodine, ferric chloride, ceric sulfate, silver oxide, lead dioxide, potassium ferricyanide and hydrogen peroxide[15] were all completely ineffective under the conditions used. Permanganate was effective in alkaline, neutral and weakly acid solutions; in strongly acid solution, weakly active or inactive products were obtained. Manganese dioxide, as would be expected if oxidation were involved, produced increased activity only in acid solutions. Only those oxidants are effective which are known in other cases to attack side chains of aromatic molecules.

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

(12) Harris, Heyl and Folkers, *J. Biol. Chem.*, **154**, 315 (1944).

(13) Bergel and Cohen, *J. Chem. Soc.*, 795 (1941).

(14) Snell and Rannefeld, in press.

(15) Other investigators (Carpenter, *et al.*, *Proc. Soc. Expt. Biol. Med.*, **54**, 123 (1943), and *Arch. Biochem.*, **3**, 375 (1944); Scott, *et al.*, *J. Biol. Chem.*, **154**, 713 (1944)) have reported increased activity of pyridoxine for *L. casei* following oxidation with hydrogen peroxide at room temperature. In some cases, at least (Carpenter, *et al.*, *op. cit.*), manganese dioxide was used to catalyze decomposition of hydrogen peroxide preliminary to assay. Since manganese dioxide itself produces the effect at room temperature it is possible that some of the observed effects were produced by this agent, rather than by hydrogen peroxide. The reported ineffectiveness of permanganate in producing the change (Carpenter, *et al.*, *op. cit.*) was likely due to use of an excess of this reagent, with consequent oxidation beyond the aldehyde stage.

TABLE IV

EFFECT OF OXIDIZING AGENTS ON ACTIVITY OF PYRIDOXINE FOR *S. fecalis*[a]

| Experiment number | Oxidizing agent Formula | Amount used, mg. | Pyridoxine hydrochloride, mg. | Total volume, cc. | Temp., °C. | Time, minutes | Potency (pyridoxine hydrochloride = 1.0) |
|---|---|---|---|---|---|---|---|
| 1 | $KMnO_4$ | 0.3 | 1.0 | 1.1 | 25 | 30 | 61 |
| | $KMnO_4$ | 1.0 | 1.0 | 2.0 | 25 | 30 | 156 |
| | $KMnO_4$ | 10.0 | 1.0 | 2.0 | 25 | 30 | 30 |
| 2 | $K_2Cr_2O_7$ | 0.1 | 1.0 | 1.0 | 100 | 15 | 34 |
| | $K_2Cr_2O_7$ | 1.0 | 1.0 | 1.0 | 100 | 15 | 116 |
| | $K_2Cr_2O_7$ | 10.0 | 1.0 | 1.0 | 100 | 15 | 51 |
| | $H_2O_2$ (30%) | 1.0 | 1.0 | 1.0 | 100 | 15 | 1.0 |
| | $H_2O_2$ (30%) | 10.0 | 1.0 | 1.0 | 100 | 15 | 1.0 |
| | $CaOCl_2$ | 10.0 | 1.0 | 1.0 | 100 | 15 | 1.0 |
| | I[b] | 3.0 | 1.0 | 1.0 | 100 | 15 | 1.0 |
| | $FeCl_3$ | 5.0 | 1.0 | 1.0 | 100 | 15 | 1.0 |
| 3 | $MnO_2$ | 5.0 | 1.0 | 0.5 | 100 | 15 | 148 |
| | $MnO_2$ | 25.0 | 1.0 | 25.0 | 25 | 240 | 25 |
| | $Ce(SO_4)_2$ | 10.0 | 1.0 | 1.0 | 100 | 15 | 1.0 |
| | $Ag_2O$[d] | .. | 1.0 | 1.0 | 100 | 15 | 1.0 |
| 4 | $PbO_2$ | 10.0 | 1.0 | 1.0 | 100 | 15 | 1.0 |
| | $K_3Fe(CN)_6$ | 10.0 | 1.0 | 1.0 | 100 | 15 | 1.0 |
| | $K_2CrO_4$ | 1.0 | 1.0 | 1.0 | 100 | 15 | 100 |
| | $CuSO_4$[e] | 5.0 | 1.0 | 2.0 | 100 | 15 | 1.0 |
| | $KClO_3$ | 1.0 | 1.0 | 0.5 | 100 | 15 | 9.0 |

[a] The *p*H of the reaction mixture was that produced by mixing the reactants in unbuffered solution, unless otherwise noted. [b] In 1 cc. 5 *N* sodium hydroxide. [c] 0.5 cc. 3 *N* hydrochloric acid added. [d] Freshly prepared from silver nitrate, and used in amount equivalent to 10 mg. of silver nitrate. [e] As Fehling solution.

This suggested that oxidation of one or more of the three side chains of pyridoxine was responsible for producing the compound with increased activity.

A number of experiments were designed to determine the nature of the active oxidation product. These are summarized below.

Ten mg. of pyridoxine hydrochloride and 10 mg. of manganese dioxide were mixed in 2 cc. of water and heated at 100° for fifteen minutes. The solution was diluted to 10 cc., centrifuged, and 1-cc. portions treated as follows

a. 1 cc. was incubated five hours at room temperature with 3 mg. ammonium chloride.
b. 1 cc. was incubated five hours at room temperature with 3 mg. sodium cyanide.
c. 1 cc. was incubated five hours at room temperature with 3 mg. ammonium chloride plus 3 mg. sodium cyanide.

An exactly similar set of tubes, each containing an amount of the crude active amine (obtained by ammonia treatment of pyridoxine tribenzoate, followed by alkaline hydrolysis) equivalent to 1 mg. of pyridoxine hydrochloride, was set up. These were to serve both as a control, to be certain the cyanide added in the above experiments would not prove toxic to the test organisms, and to test the stability of the active amine produced by ammonia treatment of pyridoxine esters toward the above treatments. Results are shown in Table V.

In a second set of experiments, stability to incubation with acetaldehyde and acetone was determined. 1-cc. portions (equivalent to 1 mg. of pyridoxine hydrochloride) of the oxidized pyridoxine were again used. These were treated as follows

a. 1 cc. + 1 cc. of 5 *N* sodium hydroxide, room temperature for four hours.
b. 1 cc. + 1 cc. of 5 *N* sodium hydroxide + 0.1 cc. of acetaldehyde, room temperature for four hours.
c. 1 cc. + 1 cc. of 5 *N* sodium hydroxide + 1.0 cc. of acetone, room temperature for four hours.
d. 1 cc. + 1 cc. of acetone (no alkali), room temperature for four hours.

Results are given in Table V.

TABLE V

STABILITY OF ACTIVE "OXIDIZED PYRIDOXINE" AND "AMINATED PYRIDOXINE" TO VARIOUS TREATMENTS

| Product treated | Treatment | Potency (pyridoxine hydrochloride = 1.0) *S. fecalis* | *L. casei* |
|---|---|---|---|
| $MnO_2$ oxidized pyridoxine | None | 48 | 44 |
| | Incubate with $NH_4Cl$ | 48 | 42 |
| | Incubate with NaCN | 13 | 10 |
| | Incubate with NaCN + $NH_4Cl$ | 3 | 5 |
| Aminated pyridoxine | None | 142 | 3.2 |
| | Incubate with $NH_4Cl$ | 142 | .. |
| | Incubate with NaCN | 140 | .. |
| | Incubate with NaCN + $NH_4Cl$ | 145 | .. |
| $MnO_2$ oxidized pyridoxine | None | 60 | .. |
| | Incubate with alkali | 58 | .. |
| | Incubate with alkali + acetaldehyde | 0 | .. |
| | Incubate with alkali + acetone | 0 | .. |
| | Incubate with acetone (no alkali) | 66 | .. |
| $MnO_2$ oxidized pyridoxine | None | 140 | .. |
| | Filtrate from phenylhydrazine treatment | 0.0 | .. |
| | Insoluble phenylhydrazone | 14.2 | .. |
| | Insoluble phenylhydrazone, $H_2SO_4$ hydrolyzed | 38.0 | .. |

A number of points are clearly illustrated. The oxidation product is highly active for both *S. fecalis* and *L. casei*, in contrast to the amine, which is highly active for *S. fecalis*, but much less active for *L. casei*. The oxidation product, but not the amine, is destroyed by incubation with cyanide, or cyanide plus ammonium chloride. The oxidized product is likewise completely destroyed by incubating with alkali and acetaldehyde or acetone, but not by incubating with alkali alone or acetone alone. These reactions are conclusive evidence for the presence of a carbonyl grouping in the active oxidized product. If derived

by oxidation of a side chain present in pyridoxine, this could only be an aldehyde group.

The presence of an aldehyde group was further demonstrated by the formation of an insoluble phenylhydrazone. To 1 cc. of phenylhydrazine in 10 cc. of water, glacial acetic acid was added dropwise until solution just occurred. A neutral solution of oxidized pyridoxine, prepared from 500 mg. of pyridoxine hydrochloride by refluxing for one-half hour with 500 mg. of manganese dioxide in 10 cc. of 0.5 *N* sulfuric acid, was added. The precipitate formed was filtered out, and washed well with water. It was dissolved in ethanol, and a portion heated at 100° with 7 *N* sulfuric acid. Filtrate, phenylhydrazone, and acid-treated phenylhydrazone were assayed with *S. fecalis*. The phenylhydrazone showed low activity; this was somewhat increased by acid-treatment, but regeneration of the original activity was not complete. The filtrate was inactive. A control run with untreated pyridoxine yielded no precipitate with phenylhydrazine. The phenylhydrazone was not further purified.

## Discussion

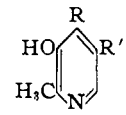
The above data show that an amine with greatly increased activity for *S. fecalis* and with somewhat increased activity for *L. casei* is formed when pyridoxine or pyridoxine esters are heated with ammonia. Examination of the pyridoxine molecule indicates that the product formed could originate only by replacement of one or more of the hydroxyl groups of pyridoxine by amino groups. Seven such amines are possible.

By heating with certain oxidizing agents, an aldehyde is formed from pyridoxine which has greatly increased growth-promoting activity for both *L. casei* and *S. fecalis*. The aldehyde must be formed by oxidation of one or more of the three side chains of pyridoxine to an aldehyde group. No data were obtained to indicate the state of oxidation of the remaining groups. Somewhat more than twenty aldehydes derived from pyridoxine in this manner are theoretically possible.

From what is known of structural specificity in relation to biochemical functioning, both within and outside of the field of vitamins, it seemed almost certain that pyridoxine, the active amine, and the active aldehyde must be interconvertible by processes which are known to occur in living organisms. Such relationships between alcohols, aldehydes and amines are well known, and are summarized by the scheme below

$$\underset{\text{I}}{RCH_2OH} \rightleftharpoons \underset{\text{II}}{RCHO} \rightleftharpoons \underset{\text{III}}{RCH{=}NH} \rightleftharpoons \underset{\text{IV}}{RCH_2NH_2}$$

It seemed reasonable to expect, therefore, that the active compounds might be interconvertible in a similar manner in the organism. Organisms such as *S. fecalis* and *L. casei* for which pyridoxine has very low activity, would effect the change from I to II with difficulty according to this view. Acceptance of such a hypothesis would require that the amino group or groups in the active amine correspond in position to the aldehyde group or groups in the active aldehyde, and that the remainder of the molecule be identical in both cases. Thus replacement of the phenolic group of pyridoxine by an amino group is excluded, since no corresponding aldehyde is possible. Similarly, oxidation of the methyl group in the 2-position of pyridoxine should not be involved in production of an active aldehyde, since ammonia would not, in all likelihood, attack such a group. These considerations limit the groups which may be involved to two, namely, the hydroxymethyl groups present in the 4 and 5 positions; and correspondingly reduce the number of possible amines and aldehydes to three each. These are



| | | |
|---|---|---|
| I | R = $CH_2NH_2$ | R′ = $CH_2OH$ |
| II | R = $CH_2OH$ | R′ = $CH_2NH_2$ |
| III | R = $CH_2NH_2$ | R′ = $CH_2NH_2$ |
| IV | R = CHO | R′ = $CH_2OH$ |
| V | R = $CH_2OH$ | R′ = CHO |
| VI | R = CHO | R′ = CHO |

Decision as to the correct formula for the active products could be obtained by synthesis of a maximum of four compounds. Synthesis of the three amines, for example, should yield one active product; synthesis of the corresponding aldehyde should then yield the active aldehyde.

The essential validity of this line of reasoning has been confirmed by synthesis of the active products, which has been briefly announced,[4,12] and which is described in detail in an accompanying report.[16] The active amine, I, has been named pyridoxamine, and the active aldehyde, IV, pyridoxal.[4] Whether these two substances are fully responsible for the pseudopyridoxine activity of natural materials is not known. Certain relationships between them and the naturally occurring material will be described in a later publication.

The fact that these substances are much more active for lactic acid bacteria than is pyridoxine indicates that they are more closely related to the catalytically effective substance derived in the body from pyridoxine than is pyridoxine itself, and that they should prove interchangeable with pyridoxine in all organisms which utilize the latter readily. This is contrary to the view expressed by Carpenter, *et al.*,[15] who concluded that the active oxidation product derived from pyridoxine (pyridoxal) is inactive for animals and yeast. Their experiments were not conclusive, however, and permit only the conclusion that the oxidation product was not significantly more active for yeast or animals than the pyridoxine from which it was derived. An account of the activity of these substances for various organisms has been given[4,14]

## Summary

Treatment of pyridoxine with ammonia gives, in low yield, a product which is much more active in promoting growth of *S. fecalis* than is pyridoxine. The amount of this substance formed is greatly increased when pyridoxine esters are treated with ammonia. This action of ammonia

(16) Harris, Heyl and Folkers, THIS JOURNAL, 66, 2088 (1944). Paper II of this series.

is specific. The product formed is destroyed by nitrous acid, and appears to be an amine.

Treatment of pyridoxine with certain oxidizing agents produces a substance with greatly increased growth-promoting power for *S. fecalis* and *L. casei*. This substance is destroyed by incubating with sodium cyanide, or with alkaline acetone or acetaldehyde. It forms a phenylhydrazone, and appears to be an aldehyde.

Adoption of a theory permitting interconversion of pyridoxine, the amine and the aldehyde by a process similar to those known to occur physiologically reduced the possible structures for the latter two compounds to three each. Synthesis of a maximum of four compounds should furnish the active amine and aldehyde. The successful accomplishment of this has been noted elsewhere.[4,12]

AUSTIN, TEXAS RECEIVED SEPTEMBER 30, 1944

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

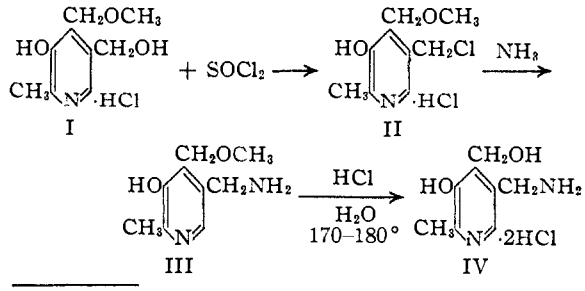
# The Vitamin $B_6$ Group. II. The Structure and Synthesis of Pyridoxamine and Pyridoxal[1]

BY STANTON A. HARRIS, DOROTHEA HEYL AND KARL FOLKERS

In a previous paper,[2] the presence in natural materials of a pyridoxine-like substance which has an extremely high growth promoting power for certain lactic acid bacteria was reported. Snell[3a,3b] showed later that procedures involving amination or oxidation of pyridoxine resulted in substances having this greatly increased activity. Further study indicated that the active product obtained by amination was an amine and the one from oxidation an aldehyde, and that there were three possible structures for each of these compounds.

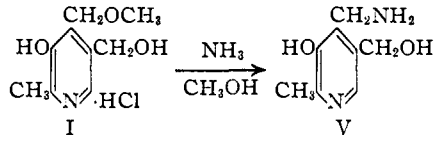
In a recent communication[4] it was stated that as a result of collaboration with Dr. Snell we had succeeded in synthesizing several of his proposed compounds including both an active amine and an active aldehyde, and had proved that these were 2-methyl-3-hydroxy-4-aminomethyl-5-hydroxymethylpyridine and 2-methyl-3-hydroxy-4-formyl - 5 - hydroxymethylpyridine, respectively. The former was named pyridoxamine and the latter pyridoxal.[5]

The synthesis of 2-methyl-3-hydroxy-4-hydroxymethyl-5-aminomethylpyridine, IV, was carried out by the following reactions, which leave no doubt concerning the structure of the final product.




The methoxy derivative, I,[6] was converted by means of thionyl chloride to the methoxy chloro compound, II,[7] and this was aminated at room temperature with ammonia to give the methoxy amino derivative, III, which was isolated as the dihydrochloride and then hydrolyzed under pressure to the hydroxy amino derivative, IV. The latter compound, 2-methyl-3-hydroxy-4-hydroxymethyl-5-aminomethylpyridine dihydrochloride, was found to be without significant growth promoting activity[8] on several different test organisms.

This 5-aminomethyl derivative of known structure could then be compared with the amine obtained by the direct amination of pyridoxine or its derivatives. Although the direct amination of pyridoxine diacetate[6] resulted in an active amine,[3b,4] the yields were better when the 4-methoxymethyl derivative, I, was heated with ammonia and methanol in an autoclave at 140°.




The amine, V, differed in chemical properties from the previously obtained amine, IV, and was found to be highly active[5,8] in promoting the growth of *L. casei* and *S. lactis R.* The replacement of the methoxy group by the amino group and the difference between the two amines showed that the active amine was 2-methyl-3-hydroxy - 4 - aminomethyl - 5 - hydroxymethylpyridine. This structure for the active amine was anticipated by us since previous work[6,9] had shown that substitution reactions take place readily on the 4-methylene position of pyridoxine.

The aldehyde resulting from the oxidation of pyridoxine with potassium permanganate was

(1) Presented before the Organic Division of the American Chemical Society at the meeting on September 13, 1944, in New York.

(2) Snell, Guirard, and Williams, *J. Biol. Chem.*, **143**, 519 (1942).

(3) (a) Snell, *Proc. Soc. Exptl. Biol. Med.*, **51**, 356 (1942); (b) Snell, THIS JOURNAL, **66**, 2082 (1944), the first paper of this series.

(4) Harris, Heyl and Folkers, *J. Biol. Chem.*, **154**, 315 (1944).

(5) Snell, *ibid.*, **154**, 313 (1944).

(6) Harris, THIS JOURNAL, **62**, 3203 (1940).

(7) Unpublished work by Dr. R. L. Clark in this Laboratory.

(8) Snell and Rannefeld, in press.

(9) Harris, THIS JOURNAL, **63**, 3363 (1941).