[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

The Microbiological Activity of Pyridoxylamino Acids[1]

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Before the participation of vitamin B_6 in enzymatic transamination was suspected, it was commonly assumed that transamination involved formation of a Schiff base between the amino acid–keto acid pair, with subsequent shift of the double bond and hydrolysis.[2] Karrer[3] pointed out that shift in the double bond might also be effected by hydrogenation of the Schiff base formed initially, followed by dehydrogenation in the opposite sense. The subsequent discovery of the participation of vitamin B_6 in chemical[4] and biological transamination reactions[5,6] and isolation of single enzymes which could carry out the entire reaction[7,8,9] greatly lessens the possibility that such intermediate hydrogenation and dehydrogenation occur, and shifts interest from the Schiff bases of the amino acids with keto acids, to those formed by condensation of amino acids with pyridoxal or its phosphate, the coenzyme of transamination.

Before the latter advances in our knowledge of transamination were made, it appeared worth while to synthesize several of the *pyridoxylamino acids* which would result from reduction of the Schiff bases formed between pyridoxal and amino acids, and to examine these compounds for physiological activity, either as vitamins or as antivitamins. Synthesis of twenty such compounds is reported in an accompanying paper.[10] The present report in this collaborative study deals with the activity of these compounds for various microörganisms. A separate report[11] will discuss the limited activity of certain of these compounds for animals.

For testing, all compounds were dissolved and dilutions made in cooled, freshly autoclaved (air-free) water. Variable amounts of these solutions were added to tubes which had been previously autoclaved with sufficient water to make the total volume 5 cc. Five cc. of the sterile culture medium was then added aseptically to each tube. Previouslv described vitamin B_6-free media and conditions were used for testing with the following organisms: *Lactobacillus casei*,[12,13] *Saccharomyces carlsbergensis* 4228,[14,15] *Streptococcus faecalis* R,[16] and *Neurospora sitophila* 299.[17]

Eighteen pyridoxylamino acids of type formula I, derived from the following amino acids, were tested: DL-alanine, DL-aspartic acid, L-asparagine, DL-glutamic acid, L-glutamic acid, glycine, DL-isoleucine, DL-leucine, L-leucine, L-lysine, DL-methionine, DL-norleucine, DL-phenylalanine, DL-serine, DL-threonine, DL-tryptophan, L-tyrosine, and DL-valine.

For *Saccharomyces carlsbergensis*, *Streptococcus faecalis* and *Lactobacillus casei*, none of these compounds was more than 0.5% as active on the weight basis as pyridoxal hydrochloride; twelve of the eighteen compounds were less than 0.1% as active as pyridoxal hydrochloride. Activities of several of the compounds are given as examples in Table I. Little significance can be attached to activities of this magnitude, since they might arise either from residual traces of the pyridoxal used in preparing the compounds, or from a slight and purely chemical degradation to pyridoxal or pyridoxamine during the incubation period (see below), and not from actual utilization of the compounds *per se* by the test organisms. Similarly, none of the compounds seemed to have significant antivitamin activity, since their addition in sufficient quantities to vitamin B_6-free media permitted growth of each of these three organisms to occur. For *Neurospora sitophila*, various compounds in this group exhibited growth-promoting activities varying from 0.1% (pyridoxyl-DL-glutamic acid) to 1.0% (pyridoxyl-DL-tryptophan) that of pyridoxal hydrochloride. Activities of this latter magnitude probably indicate limited ability of the mold to utilize certain of these compounds, especially in view of the fact that this same compound showed only a trace of activity for the other assay organisms.

Very different results were obtained if dilutions of these compounds suitable for assay were prepared in ordinary distilled water, autoclaved, and then assayed by the above procedures. Under these conditions, most of the compounds tested showed high activity, approaching in many instances that of equimolar quantities of pyridoxal. Some illustrative results are given in Table I. Activity under these conditions is attributed to

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(2) Herbst, "Advances in Enzymology," Interscience Press, New York, N. Y., 1944, Vol. IV, p. 75.

(3) Karrer, Koenig and Legler, *Helv. Chim. Acta*, **24**, 127 (1941).

(4) Snell, THIS JOURNAL, **67**, 194 (1945).

(5) Schlenk and Snell, *J. Biol. Chem.*, **167**, 425 (1945).

(6) Umbreit, O'Kane and Gunsalus, *J. Bact.*, **51**, 576 (1946).

(7) Green, Leloir and Nocito, *J. Biol. Chem.*, **161**, 559 (1945).

(8) Schlenk and Fisher, *Arch. Biochem.*, **12**, 69 (1947).

(9) O'Kane and Gunsalus, *J. Biol. Chem.*, **170**, 425 (1947).

(10) Heyl, Harris and Folkers, THIS JOURNAL, **70**, 3429 (1948).

(11) Emerson, to be published.

(12) Snell and Rannefeld, *J. Biol. Chem.*, **157**, 475 (1945).

(13) Rabinowitz, Mondy and Snell, *J. Biol. Chem.*, **175**, 147 (1948).

(14) Rabinowitz and Snell, *Anal. Chem.*, **19**, 277 (1947).

(15) Atkin, Schultz, Williams and Frey, *Ind. Eng. Chem., Anal. Ed.*, **15**, 141 (1943).

(16) Rabinowitz and Snell, *J. Biol. Chem.*, **169**, 631 (1947).

(17) Stokes, Larsen, Woodward and Foster, *J. Biol. Chem.*, **150**, 17 (1943).

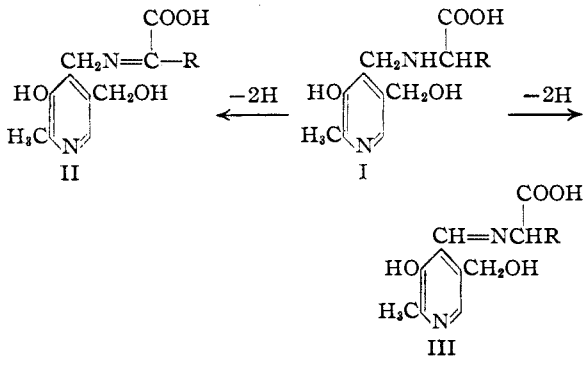
TABLE I

ACTIVITY OF PYRIDOXYLAMINO ACIDS IN REPLACING VITAMIN B_6 FOR VARIOUS MICROÖRGANISMS

Compound	Autoclaved	Activity (Pyridoxal hydrochloride = 1.0) *Lactobacillus casei*	*Saccharomyces carlsbergenesis*	*Streptococcus faecalis*	*Neurospora sitophila*
Pyridoxyl-DL-alanine	No	0.0008	<0.0006	<0.0003	0.004
tryptophan	No	< .0003	< .0003	< .0003	.01
phenylalanine	No	.0010	< .0003	< .0003	.002
valine	No	< .0003	.0008	< .0003	.004
leucine	No	.0010	< .0003	< .0003	.002
phenylalanine	In water, no antioxidants[a]	.18	.40	.35	...
valine	In water, no antioxidants[a]	.15	.42	.70	...
leucine	In water, no antioxidants[b]	.42	.57	.60	...
leucine	With ascorbic acid[c]	.020	< .040	.....	...
leucine	With cysteine[c]	.005	< .040	.....	...
leucine	With basal medium[d]	.006	.040	.04	...
Pyridoxal hydrochloride	With basal medium[d]	.16[e]	.77	.....	...

[a] 0.003 to 0.10γ of compound in 1 to 5 cc. of water, autoclaved at 120° for five minutes. [b] 0.003 to 0.05γ of compound in 0.5 to 2.5 cc. of water, autoclaved at 120° for fifteen minutes. [c] As in footnote [b], but with 1 mg. of added antioxidant per tube. [d] 0.003 to 0.05γ of compound in 5 cc. of basal medium (1% glucose), autoclaved at 120° for fifteen minutes. [e] The low result obtained with *L. casei* when pyridoxal is autoclaved with the basal medium has been shown elsewhere to result from transamination to pyridoxamine, which is inactive for *L. casei* but highly active for *Saccharomyces carlsbergensis* (see references 4 and 12).

dehydrogenation (by traces of oxygen present in the distilled water and during preliminary phases of autoclaving) of the pyridoxylamino acids (I) in the following manner



with subsequent hydrolysis of the Schiff bases (II and III) to yield pyridoxamine or pyridoxal, respectively. As would be expected from this formulation, activity of the hydrolytic products for *S. carlsbergensis* and *S. faecalis*, which respond to both pyridoxal and pyridoxamine, is greater than for *L. casei*, which responds only to pyridoxal.[11] That oxidative degradation of the compounds is actually involved is shown by the fact that the products show little or no increase in activity when autoclaved in the presence of antioxidants such as ascorbic acid, cysteine or the complete basal media for the various test organisms, each of which contains excess glucose (Table I). This eliminates the possibility that a purely hydrolytic breakdown of the secondary amine (I) to pyridoxine or pyridoxamine occurs, followed by partial oxidation to pyridoxal. In the latter case, products heated with antioxidants should show high activity for yeast (for which both pyridoxine and pyridoxamine are fully active[11]); actually, they do not. It is obvious that increases in activity of the magnitude shown would be expected only when extremely dilute solutions of the compounds are autoclaved in water containing air, since the amount of oxygen present is small, and since only under these conditions would the active reaction products constitute an appreciable fraction of the total material present. Somewhat similar changes produced by dissolved air when extremely dilute solutions of other physiologically active compounds are heated, and which may produce serious interpretive errors, have been noted previously.[18]

The microbiological activity of several additional compounds related in structure to vitamin B_6 or the pyridoxylamino acids, but which do not correspond to general formula I, was determined. Pyridoxylideneaniline was, within experimental error, as active as equimolar amounts of pyridoxal hydrochloride. This was true also of other Schiff bases tested, and lends support to the explanation previously given for the pronounced activity of the pyridoxylamino acids when tested under conditions which permit oxidative hydrolysis. Full activity would be expected of these compounds, since spontaneous hydrolysis, as evidenced by the gradual disappearance of the yellow color, occurs in water solution.

The thiazolidinecarboxylic acid formed from L-cysteine and pyridoxal (2-(2-methyl-3-hydroxy-5-hydroxymethyl - 4 - pyridyl) - 4 - thiazolidinecarboxylic acid) likewise showed activity equivalent to equimolar amounts of pyridoxal for all organisms. It has been shown with similar compounds formed between cysteine and other aldehydes[19,20] that an equilibrium exists in water solutions be-

(18) Cunningham and Snell, *J. Biol. Chem.*, **158**, 491 (1945).
(19) Schubert, *J. Biol. Chem.*, **111**, 671 (1935); **114**, 341 (1936).
(20) Ratner and Clarke, THIS JOURNAL, **59**, 200 (1937).

tween the thiazolidinecarboxylic acid on the one side, and cysteine and aldehyde on the other. Thus irreversible removal of one product of the equilibrium will eventually result in complete dissociation of the compound. It appears likely that the observed activity of this compound for the various assay organisms may result solely from this process, which would be accelerated by utilization of the pyridoxal for growth and perhaps by other ingredients of the medium. Active utilization of the compound *per se* by the various organisms is, of course, also a possibility.

In contrast to the activity of these products, the condensation product formed between histidine and pyridoxal (4-(2-methyl-3-hydroxy-5-hydroxymethyl - 4 - pyridyl) - 1 - imidazo(c)tetrahydropyridine-6-carboxylic acid) was essentially inactive for all organisms. Pyridoxyl-β-alanine was also inactive.

Summary

A series of pyridoxylamino acids and some related structures were tested for vitamin and antivitamin activities against four microörganisms of diverse types. For *L. casei*, *S. faecalis* and *S. carlsbergensis*, these compounds were essentially inactive. Although growth-promoting activity was shown at high concentration levels, the magnitude of this action was such that it could well be attributed to trace impurities, or to purely chemical breakdown during incubation in the medium. For *N. sitophila*, the compounds had somewhat greater activity which, however, was still of a low order. No significant "antivitamin" activity was demonstrated with these organisms.

When autoclaved in extremely dilute solutions containing dissolved air and subsequently tested, the compounds show high activity, approaching that of equimolar quantities of pyridoxal for all test organisms. This is ascribed to their oxidation to the corresponding Schiff bases, with subsequent hydrolysis to yield pyridoxal or pyridoxamine. The presence of ascorbic acid, cysteine or other antioxidants prevents this change.

True Schiff bases of pyridoxal and the thiazolidinecarboxylic acid formed by condensation of pyridoxal with cysteine exhibit high growth-promoting activity for all organisms. In both cases, such activity probably results from spontaneous dissociation, which proceeds to completion as the pyridoxal formed is utilized for growth.

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The Chemistry of Vitamin B_6. V.[1] Conversion of Pyridoxine to the Lactone of 4-Pyridoxic Acid[2]

BY DOROTHEA HEYL

Several syntheses of the lactone of 2-methyl-3 - hydroxy - 4 - carboxy - 5 - hydroxymethylpyridine have been described.[3,4,5] Direct oxidation of pyridoxine produces the lactone[3,4] in poor yield and is not convenient for the preparation of the lactone in quantity. Stepwise conversion of pyridoxine to the lactone of 4-pyridoxic acid has now been found to be more successful.

Oxidation of pyridoxine to pyridoxal is accomplished in better yield with manganese dioxide and sulfuric acid than with potassium permanganate.[3] Pyridoxal was isolated as the oxime (II), which was then acetylated and dehydrated to the diacetyl nitrile IV. By variation in the length of reaction time, it was found that the acetylation and dehydration could be accomplished either separately, with the isolation of the intermediate triacetyl oxime III, or in one operation. The diacetyl nitrile IV was hydrolyzed in alkaline solution to 4-pyridoxic acid (V), which was isolated by acidification. Lactonization occurred when the acid was refluxed with alcoholic hydrogen chloride.

The lactone VI was converted to the hydrochloride by treatment with saturated alcoholic hydrogen chloride. Pure 4-pyridoxic acid was prepared from the lactone by saponification.

Removal of the acetyl group from the phenolic hydroxyl group of the diacetyl nitrile IV by treatment with alcoholic sodium ethoxide resulted in the production of 2-methyl-3-hydroxy-4-cyano-5-acetoxymethylpyridine (VII).

Thionyl chloride was also used to dehydrate pyridoxal oxime (II). The resulting 2-methyl-3 - hydroxy - 4 - cyano - 5 - chloromethylpyridine (VIII) was hydrolyzed by water to 2-methyl-3-hydroxy - 4 - carbamyl - 5 - hydroxymethylpyridine (IX).

Experimental

Pyridoxal Oxime (II).—In 1.5 l. of water in a 3-l. round-bottomed flask equipped with a mechanical stirrer, 102.8 g. of pyridoxine hydrochloride (I) was dissolved. In this

(1) For the preceding paper of this series, see Harris, THIS JOURNAL, **63**, 3363 (1941).

(2) 4-Pyridoxic acid is the name designated for 2-methyl-3-hydroxy-4-carboxy-5-hydroxymethylpyridine by Huff and Perlzweig (ref. 4). The name "β-pyracin" given to the lactone by Scott, Norris, Heuser and Bruce (*J. Biol. Chem.*, **158**, 291 (1945)) was later transferred to the acid by Daniel, Scott, Norris and Heuser (*ibid.*, **160**, 265 (1945)).

(3) Harris, Heyl and Folkers, THIS JOURNAL, **66**, 2088 (1944).

(4) Huff and Perlzweig, *J. Biol. Chem.*, **155**, 345 (1944).

(5) Scott, Norris, Heuser and Bruce, THIS JOURNAL, **67**, 157 (1945).