PYRIDOXINE NUTRITION OF LACTIC ACID BACTERIA[1]

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Received for publication March 9, 1942

Previous reports from this laboratory have shown that pantothenic acid is essential for the growth of every species of lactic acid bacteria tested (Snell *et al.*, 1937). It has also been shown that while some species of lactic acid bacteria require riboflavin for growth, several do not (Snell and Strong, 1939). In a study of the activity of certain synthetic flavins, a close correlation was found to exist between the rat assay and the bacterial test (Snell and Strong, 1939). Vitamin B_6 (pyridoxine) has been shown to be necessary for the growth of certain lactic acid bacteria (Möller, 1938). Möller has shown that 2:4-dimethyl-3-hydroxy-5-hydroxymethyl pyridine has a very small vitamin B_6 activity and that 2,4,5-trimethyl-3-hydroxypyridine has no activity when tested with *Streptobacterium plantarum* 10S (Möller *et al.*, 1939).

The objectives of this present study were, first, to examine the vitamin B_6 requirements of several species of lactic acid bacteria and, second, to determine the activity of some vitamin B_6 analogues for *Lactobacillus casei*[2] with the intention of comparing the observed results with the reported antidermatitic activity of these compounds on rats.

CULTURE MEDIUM

The basal medium used was such that maximum acidity could be produced by the organism, *Lactobacillus casei*, when sufficient pyridoxine was added. The composition of the medium was as follows:

Acid-hydrolyzed alcohol-extracted casein	0.5 per cent
Glucose	1.0 per cent
Sodium acetate	0.6 per cent
Tryptophane	0.01 per cent
Cystine	0.01 per cent
Riboflavin	0.01 mg. per cent
Nicotinic acid	0.02 mg. per cent
Pantothenic acid	0.02 mg. per cent
Acid ether extract of Galen B	5.0 mg. per cent
Alcohol-extracted solubilized liver	2.0 mg. per cent
Inorganic salts (K_2HPO_4 0.5 gm.; KH_2PO_4 0.5 gm.; $MgSO_4 \cdot 7H_2O$ 0.2 gm.; NaCl 0.01 gm.; $FeSO_4 \cdot 7H_2O$ 0.01 gm.; $MnSO_4 \cdot 3H_2O$ 0.01 gm.; in 1000 ml. of medium.)	

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

[2] This is the organism which has been widely used for the determination of riboflavin and pantothenic acid and recently adapted for biotin assay. It has usually been called *L. casei* ϵ but also has been designated as *L. casei* and *L. helveticus*. In accordance with the views of several bacteriologists who have been consulted and in accordance with the report of Tittsler *et al.* (1942), the name *Lactobacillus casei* American Type Culture Collection No. 7469 is used in this paper.

To free the casein of vitamin B_6 activity it was given the following treatment. One hundred grams of Labco casein were ground to pass through a No. 80 mesh sieve and then refluxed 3 times, 3 hours each time, twice with 350 ml. of absolute alcohol and finally with 350 ml. of 95 per cent ethanol. The casein was then dissolved in 3 liters of dilute ammonium hydroxide and precipitated with hydrochloric acid, dried and ground to pass through a No. 100 mesh sieve and refluxed once more for 3 hours with 350 ml. of absolute ethanol and once more for 3 hours with 350 ml. of 95 per cent ethanol. The yield was around 60 grams. To prepare the casein hydrolysates 20 grams of the alcohol-extracted casein were autoclaved at 20 lbs. pressure with 800 ml. of 10 per cent HCl for 3 hours. The HCl was removed *in vacuo*, the solution was adjusted to pH 6.8 and then diluted to make a 5 per cent concentration of hydrolyzed casein.

The acid ether extract of Galen B[3] was made by extracting a 20 per cent solution of Galen B at pH 2.5 with peroxide-free ether in a Kutcher-Steudel extractor for 72 hours, changing the ether every 24 hours. The ether extract was concentrated to dryness and then made up to a concentration of 5 mg. per ml.

Five grams of solubilized liver[4] were refluxed with 100 ml. of absolute ethyl alcohol for 3 hours and then filtered. The extraction was repeated 3 times. The extracted solubilized liver was dried and dissolved as needed.

CULTURES

The cultures were as follows: *Lactobacillus arabinosus* 17-5; *Lactobacillus casei; Lactobacillus delbrückii* 3; *Lactobacillus pentosus* 124-2; *Lactobacillus lactis* Bl-1; and *Leuconostoc mesenteroides* P-60. The organisms were carried as stab cultures in yeast water glucose agar.

TECHNIQUE OF BIOLOGICAL TESTING

The inocula were prepared as follows: The organisms were grown in 0.5 per cent peptone, 0.6 per cent sodium acetate, 1 per cent glucose, inorganic salts medium. Transfers were made every 24 hours. For inocula, second or third transfers were used. The cells in 10 ml. of a 24-hour culture were centrifuged down, suspended in 10 ml. sterile 0.9 per cent saline solution, and 0.1 ml. of this suspension was added to 80 ml. of sterile 0.9 per cent saline solution and thoroughly mixed. 0.3 ml. of this last suspension was used as inoculum for each tube. This inoculum was used in all experiments unless otherwise indicated.

The growth tests were carried out in test tubes containing 10 ml. of medium at pH 6.8. The basal medium was first made up at 1.25 times the above concentration (8 ml. per tube) and the test material was incorporated in a 2 ml.

[3] We are indebted to the Galen Co., Inc., Berkeley, California for the gift of Galen B.

[4] The solubilized liver fraction is that portion of an aqueous liver extract precipitated from solution by addition of ethanol to 70 per cent concentration, then rendered water soluble by enzyme action. We wish to thank Dr. David Klein, of the Wilson Laboratories, Chicago, for this preparation.

addition. The tubes were autoclaved at 15 lbs. pressure for 15 minutes, cooled and inoculated.

Cultures of *L. mesenteroides*, *L. arabinosus* and *L. pentosus* were incubated at 25°C., the other three bacteria were incubated at 37°C. Growth observations were made by turbidity readings with the Evelyn photoelectric colorimeter. Fermentation was followed by titration of acid produced after 3 days growth.

In a comparison of the various tables it will be found that *L. casei* does not give a standard response to a given level of pyridoxine. It has been impossible to control this variation to any great extent. However, it is known to be associated with age of culture, number of subcultures from the stock culture and the amount of pyridoxine present in the culture medium. Data for each table are taken from the same experiment. The individual experiments were repeated a number of times and gave the same relative values but the absolute values differed.

TABLE 1

Effect of size of inoculum on requirement for pyridoxine

INOCULUM	MICROGRAMS OF PYRIDOXINE PER 10 ML. OF MEDIUM				
	0	0.2	0.4	1.0	2.0
	ml. N/10 acid	*ml. N/10 acid*	*ml. N/10 acid*	*ml. N/10 acid*	*ml. N/10 acid*
A	3.5	4.6	10.2	10.6	10.2
	6.2	4.8	8.6	10.1	10.2
B	1.0	5.6	6.3	10.4	10.2
	1.0	3.5	5.4	10.2	10.4
C	0.4	0.8	2.8	9.5	10.1
	0.5	1.0	2.6	9.5	10.3

PYRIDOXINE REQUIREMENTS OF L. CASEI

The amount of pyridoxine necessary for maximum growth and acid production depends on the size of the inoculum. This was readily demonstrated by the following experiment. The cells from 10 ml. of a 24-hour culture grown in the peptone medium were centrifuged, suspended in 10 ml. of sterile saline solution, centrifuged again and resuspended in 10 ml. of saline. Inocula of three concentrations were prepared as follows: Inoculum A was prepared by diluting 0.5 ml. of the stock suspension with 10 ml. of saline, inoculum B by diluting 0.5 ml. of A with 10 ml. of saline, and inoculum C by diluting 0.5 ml. of B with 10 ml. of saline. 0.1 ml. of the respective dilutions was used for each tube. The data are summarized in table 1.

That these effects were not due to mechanical carry over with the cells was shown by additional washing of the cells with saline solution. The extra washing did not alter the results.

The effect noted in table 1 is probably due to the storage of more pyridoxine

by the cell than is necessary for its growth and function. The ability of *L. casei* to store an amount of a specific factor above the physiological requirements of the cell has also been observed in respect to biotin and the norit eluate factor. No such storage of riboflavin or pantothenic acid has been observed.

It is evident that, if the organism is to be used as an assay agent, it must be grown on a sub-optimum, if not a minimum, amount of the vitamin. This would prevent storage of the growth factor but allow the organism a sufficient amount of the factor for the life processes of the cell.

The effect of various methods of oxygen removal was studied. Some tubes were incubated in anaerobic jars, to some metallic iron was added, and to others 0.3 mg. $NaHSO_2$. It was found that the vitamin requirements were increased, in the order of the methods mentioned (table 2). The effect of $NaHSO_2$ seems to be due to oxygen removal rather than to toxicity of the compound. This is probable since at the higher levels of pyridoxine the organism

TABLE 2

Effect of oxygen removal on the pyridoxine requirement of L. casei

PYRIDOXINE ADDED	TUBES INCUBATED IN AIR	TUBES INCUBATED IN ANAEROBIC JARS	TUBES CONTAINING REDUCED IRON	TUBES CONTAINING 0.3 MG. $NaHSO_2$ PER 10 ML.
μg./10 ml.	*ml. N/10 acid/10 ml.*	*ml. N/10 acid/10 ml.*	*ml. N/10 acid/10 ml.*	*ml. N/10 acid/10 ml.*
0.1	6.8	5.6		0.8
0.2	9.0	6.2		0.8
0.4	8.6	7.2	0.2	0.8
0.8	8.9	8.4	0.25	2.2
1.2	9.9	8.7	4.7	2.0
1.6	10.1	9.0	7.1	2.0
2.0	10.0	9.7	8.5	5.0

is producing significant amounts of acid. It is unlikely that pyridoxine would function as a specific antagonist for $NaHSO_2$ if this compound were toxic.

The role that vitamin B_6 plays in cellular metabolism is not known, but it is significant that the bacterial cell requires more of the vitamin when the oxygen tension is reduced.

PYRIDOXINE REQUIREMENTS OF VARIOUS SPECIES OF LACTIC ACID BACTERIA

Requirement studies were made with tubes containing the basal medium, and tubes with 0.2 microgram of pyridoxine added per ml. of the basal medium. Each succeeding subculture was inoculated with cells taken from the preceding tube in the series. The results are shown in table 3.

It is obvious that *L. casei*, *L. delbrückii*, and *L. lactis* require pyridoxine in the media. *L. arabinosus* and *L. pentosus* grow just as well without pyridoxine as with it but the growth of *L. mesenteroides* is stimulated by addition of the vitamin.

To show the synthesis of vitamin B_6 by bacteria which grew in the basal medium the cells were centrifuged out, suspended in 10 ml. of water and auto-

lyzed under toluene. The centrifuged cell-free media were neutralized and concentrated to 10 ml. volumes. The cell autolysates and culture filtrates were tested for vitamin B_6 activity with *L. casei* as the assay organism. The ml. of acid produced furnish an index of the vitamin B_6 present (table 4). It was found that those bacteria requiring added pyridoxine did not synthesize the vitamin, whereas the others did. *L. pentosus* and *L. arabinosus* synthesized approximately the same amount of pyridoxine. *L. mesenteroides* synthesized about one-fourth as much pyridoxine as did the other organisms. This limited synthesis by *L. mesenteroides* is in accord with the earlier observation (table 3)

TABLE 3

Growth of successive subcultures of lactic acid bacteria in the presence and absence of pyridoxine
(Figures are readings on Evelyn colorimeter)

ORGANISM	NO PYRIDOXINE			0.2 MICROGRAMS PYRIDOXINE PER ML.		
	First	Second	Third	First	Second	Third
L. arabinosus 17-5	31	33	29	29	30	30
L. casei	89	91	91	26	26	30
L. delbrückii 3	88	88	88	57	60	61
L. pentosus 124-2	39	40	39	37	42	40
L. lactis Bl-1	86	79	71	49	56	53
L. mesenteroides P-60	62	62	60	55	59	54

TABLE 4

*Pyridoxine synthesis by certain lactic acid bacteria**

ORGANISM	AGE OF CULTURE	ML. OF ADDED FILTRATE OR AUTOLYZED CELL SUSPENSION					
		0.5		1.0		2.0	
		Filtrate	Cell	Filtrate	Cell	Filtrate	Cell
	hours						
L. lactis Bl-1	72	0.6	0.6	0.6	0.6	0.6	0.8
L. mesenteroides P-60	72	0.6	0.6	0.6	0.6	2.1	1.3
L. pentosus 124-2	72	2.4	0.8	7.5	1.8	10.4	4.5
L. arabinosus 17-5	72	2.4	1.5	5.7	1.9	10.4	4.7

* The figures are the ml. of $N/10$ acid produced per 10 ml. of medium in the assay culture.

that the growth of *L. mesenteroides* is stimulated by addition of the vitamin. Under the conditions of the test *L. mesenteroides* cannot synthesize pyridoxine at a rate sufficient to meet the requirements of the cell while *L. pentosus* and *L. arabinosus* can.

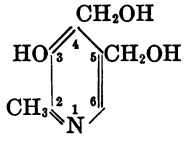
From the above data it is possible to calculate the percentage of pyridoxine present in the cell and the percentage present in the medium. In all cases the largest amount of the vitamin was present outside of the cell. This may be due to the secretion of pyridoxine into the medium or it may be due to autolysis of the cell and consequent liberation of the vitamin.

EFFECT OF PYRIDOXINE ANALOGUES ON THE GROWTH OF L. CASEI

The basal medium was supplemented with varying amounts of each of the analogues[5] and the acidity produced was compared with that when pyridoxine was added. Autoclaving solutions of compounds II and III results in hydrolysis to pyridoxine. Therefore solutions of these compounds were sterilized by filtering through a Seitz filter and adding the filtrate to the autoclaved basal medium. The remainder of the compounds were incorporated in the basal medium and autoclaved at 15 lbs. pressure for 15 minutes. The inactive compounds were tested at concentrations up to 2 micrograms per ml. The results obtained with the different pyridine derivatives are recorded in table 5.

TABLE 5

Activity of pyridoxine (vitamin B_6) derivatives

COMPOUND	ACTIVITY*
	
2-Methyl-3-hydroxy-4,5-bis(hydroxymethyl) pyridine (pyridoxine)	1.0
Derivatives of 2-methyl pyridine	
I. 3-Hydroxy-4,5-bis-(acetoxymethyl)-	0.8–1.0
II. 3-Acetoxy-4,5-bis-(acetoxymethyl)-	0
III. 3-Hydroxy-4,5-bis-(bromomethyl)-	0.6–0.8
IV. 3-Amino-4-bromomethyl-5-aminomethyl-	0
V. 3-Amino-4-hydroxymethyl-5-aminomethyl-	0
VI. 3-Amino-4-ethoxymethyl-5-aminomethyl-	0
VII. 3-Hydroxy-4-ethoxymethyl-5-hydroxymethyl-	0.3
VIII. 3-Hydroxy-4-methoxymethyl-5-hydroxymethyl-	0.3–0.4
IX. 3-Hydroxy-4,5-epoxydimethyl-	0.2–0.3
X. 3-Hydroxy-4-methyl-5-hydroxymethyl-	0.03 (?)
XI. 3-Hydroxy-4,5-dimethyl-	0
XII. Lactone of 3-hydroxy-4-hydroxymethyl-5-carboxy-	0
XIII. Lactone of 3-amino-4-hydroxymethyl-5-carboxy-	0

* Activity of on molar basis.

No analogues of pyridoxine were found to be as active as the pure vitamin. The diacetyl compound (I) was found to be nearly as active as the vitamin but the triacetyl compound (II) was inactive. In the rat assay (Unna, 1940), the di- and tri-acetates were found equally potent and of the same activity as the vitamin. Apparently the bacteria could not hydrolyze the acetate in the 3 position whereas the rat could bring about this hydrolysis. Replacement of the hydroxyls of the hydroxymethyl groups by bromine reduced the activity about 40 per cent. It was not determined whether or not the activity was due

[5] We are indebted to Dr. Karl Folkers of Merck and Company, Inc., Rahway, New Jersey, for the gift of these compounds.

to the hydrolysis of the bromines, but it was found that the solution resulting from autoclaving the compound had the same activity as the vitamin when equimolecular amounts were tested.

Replacement of the phenolic hydroxyl and the hydroxyl of the 5-hydroxymethyl group by amino groups resulted in a compound which was inactive (V), as were the derivatives of this compound (IV, VI). It may be that the lack of activity was due to the replacement of the hydroxyl in the 3 position.

When the 4-hydroxymethyl group was methylated (VIII) or ethylated (VII) there was a loss of about 70 per cent of activity. Between the two ethers a slight difference in potency was observed. A further reduction in activity resulted from the formation of an inner ether between the two hydroxyl groups in the 4 and 5 positions (IX). It is highly improbable that the organism could hydrolyze the ether linkages in these compounds. Rat assay showed much the same activity of these ethers but lesser activity of the inner ether.

When one hydroxymethyl group was replaced by a methyl group, the compound (X) was found to have very little activity, but when both the hydroxymethyl groups were replaced by methyl groups (XI) no activity was found. These results agree with those of Möller. Both of these compounds were inactive when tested with rats.

The lactone of the compound, formed by substituting a carboxyl for a hydroxymethyl group (XII), was found to be inactive for the bacteria, just as it had been found inactive for rats.

SUMMARY

Lactobacillus casei has been shown to store pyridoxine in amounts greater than are necessary for growth. The pyridoxine requirement was dependent on the oxygen tension of the medium.

Three of the six species of lactic acid bacteria investigated do not require pyridoxine for growth and acid production and they were all shown to synthesize the vitamin when cultured on a medium devoid of pyridoxine.

The response of *L. casei* to a number of pyridoxine analogues parallels rather closely the antidermatitic effect of these same compounds on rats.

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