Specificity of *Lactobacillus fermenti-36* for the Assay of Thiamine

1. FACTORS IN THE GROWTH MEDIUM INFLUENCING THE ESSENTIALITY OF THIS VITAMIN FOR THIS ORGANISM

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In a previous paper (Hoover and Jayasuriya, 1950) a satisfactory microbiological assay of thiamine in foodstuffs using *Lactobacillus fermenti*-36 was reported. It was however disturbing to find that Shankman, Camien, Block, Merrifield and Dunn (1947) had reported that whereas nicotinic and pantothenic acids were essential for this organism, thiamine was not found to be essential for short or long periods of incubation. They have suggested that the positive findings in other laboratories might be due to a relatively poor basal medium, particularly in respect of some essential amino-acids and possibly purines. Contrary to the observation of some authors, they demonstrated that in the presence of adequate amounts of methionine, adenine, guanine, xanthine and hypoxanthine, para-amino benzoic acid was not essential for *Lactobacillus arabinosus* and *Lactobacillus pentosus*. These observations led us to inquire into the adequacy of the basal medium of Hoover and Jayasuriya (1950) used in our laboratory for routine assays of thiamine.

In investigating the amino-acid requirements of *Lactobacillus fermenti*-36, Dunn, Shankman, Camien and Block (1947) found that eleven amino-acids were essential, namely, arginine, histidine, threonine, glutamic acid, valine, iso-leucine, leucine, methionine, phenyl alanine, tryptophane and tyrosine. Of these arginine, histidine and threonine were essential only for short periods of incubation. Shankman et al (1947) observed that charcoal treatment of acid hydrolysed casein used in the basal medium removed significant amounts of several amino-acids and marked quantities of phenyl alanine and tyrosine. Therefore it was necessary to investigate any amino-acid deficiency in our basal medium and clear any doubts as to whether the essentiality of thiamine for this organism arose merely from the inadequacy of amino-acids in the growth medium. It is obvious that an insufficiency of any of the eleven amino-acids in the basal medium would result in unreliable thiamine values when assaying foodstuffs containing proteins with *Lactobacillus fermenti*-36.

Experiment

The minimal amounts of the essential amino-acids in our basal medium were not known with any certainty except for tryptophane where 20 mg. were added to 100 ml. double strength medium. It might be mentioned that Dunn et al (1947) using a medium containing one-fourth the quantity of amino-acids present in their standard

medium, found that the maximal acid production was not altered by this reduction of amino-acid concentration in the case of *Lactobacillus fermenti*-36. In other words, a double strength medium containing 15 mg. per 100 ml. of each of the essential amino-acids was found to be quite sufficient for maximal acid production and therefore for optimum growth. We therefore did not consider our medium inadequate in respect to tryptophane.

It was considered likely that the sensitivity to thiamine of *Lactobacillus fermenti*-36, when grown in our basal medium, was due to the inadequacy of either a single or a number of essential amino-acids. If of a single, then an adequate supply of that amino-acid to the basal medium should enable this organism to grow optimally in the absence of thiamine ; if of a number, the organism should dispense with thiamine when the basal medium was supplemented with adequate amounts of all the essential amino-acids. To test the first, each of the essential amino-acids was added in turn to our basal medium in amounts equal to or very near to that employed by Dunn et al (1947) and the pH was adjusted to 6·5. The tubes were prepared and after inoculation incubated for 18 hours and the turbidities were compared with that produced in our standard basal medium. This was done to test the first assumption. The experimental details and results are shown in Table I.

TABLE I

Effect on the growth of Lactobacillus fermenti-36 *by the addition to a thiamin-free basal medium of each of the essential amino-acids. Values are expressed in mg. per* 100 *ml. single-strength medium.*

Medium used	*Turbidity*
Basal medium (Hoover and Jayasuriya, 1950)	14
,, + 0·5 µg. thiamine	113
,, + 50 mg. L-Arginine	14
,, + 50 mg. L-Histidine	19
,, + 50 mg. DL-Threonine	10
,, + 66 mg. L-Tyrosine	15
,, + 66 mg. DL-Methionine	13
,, + 66 mg. DL-Phenylalanine	12
,, + 66 mg. DL-Isoleucine	12
,, + 66 mg. DL-Leucine	15
,, + 66 mg. L-Glutamic acid	15
,, + 66 mg. DL-Valine	14

It was observed that the growth of *Lactobacillus fermenti*-36 was not in any way influenced by the addition of any of the essential amino-acids to our basal medium.

To test the second assumption two approaches were possible, (*a*) the basal medium could be supplemented with a mixture of all the essential amino-acids without any reference to the amounts of these acids already present in the medium, or, (*b*) a synthetic basal medium similar to that of Dunn et al (1947) could be employed. We preferred the latter approach in order to reproduce as far as possible the experimental

conditions of Dunn et al (1947). Since it is known that choline chloride, inositol, norleucine, norvaline, and hydroxy-proline found in the medium of Dunn et al (1947) have no effect on the growth of *Lactobacillus fermenti*-36, these substances and thiamine were omitted from our synthetic medium. Otherwise, the amino-acid composition of the synthetic basal medium used in our experiment was identical with that of Dunn et al (1947). The composition of the medium used is given in Table II.

TABLE II

Composition of basal synthetic medium

Constituent	*mg. /100 ml. single strength*		*Constituent*	*mg. /100 ml. single strength*	
	Our medium	*Dunn's medium*		*Our medium*	*Dunn's medium*
DL-Alanine	66·6	66·7	Uracil	1·4	1·2
Asparagine, natural	66·6	66·7	Xanthine	1·2	1·2
L-Arginine HCl	66·6	66·7	Dextrose	2000	2000
L-Cysteine HCl	66·6	66·7	Sodium acetate anhydrous	600	1200
Glycine	66·6	66·7	NH_4Cl	600	600
L-Histidine $HCl.H_2O$	66·6	66·7	KH_2PO_4	50	50
L-Hydroxyproline	—	66·7	K_2HPO_4	50	50
DL-Isoleucine	66·6	66·7	$MgSO_4.\ 7H_2O$	20	20
L-Leucine	66·6	66·7	$FeSO_4.\ 7H_2O$	1	1
DL-Lysine HCl	66·6	66·7	$MnSO_4.\ 4H_2O$	1	1
DL-Methionine	66·6	66·7	NaCl	35	35
DL-Norleucine	—	66·7	Thiamine HCl	—	0·1
DL-Norvaline	—	66·7	Pyridoxin	0·35	0·16
DL-Phenylalanine	66·6	66·7	Pyridoxamine 2HCl	0·01	0·01
L-Proline	66·6	66·7	Pyridoxal HCl	0·01	0·01
DL-Serine	66·6	66·7	DL-Calcium pantothenate	0·16	0·20
DL-Threonine	66·6	66·7	Riboflavin	0·16	0·20
DL-Tryptophane	66·6	66·7	Nicotinic acid	0·16	0·20
L-Tyrosine	66·6	66·7	Biotin	0·0005	0·0005
DL-Valine	66·6	66·7	P.A.B.	0·08	0·01
Adenine sulphate $2H_2O$	2·7	1·4	Folic acid	0·0004	0·0005
Guanine $HCl.2H_2O$	2·0	1·3	Choline chloride	—	1
			Inositol	—	2·5

Since our standard basal medium was deficient in xanthine and ammonium chloride as compared with that of Dunn et al (1947), experiments were also carried out to study any possible effects of these nutrients on the growth of the organism. The amounts of xanthine and ammonium chloride added to every 100 ml. of single strength basal medium of Hoover and Jayasuriya (1950) were 2 mg. and 600 mg. respectively.

Organism :—*Lactobacillus fermenti*-36, obtained from the National Collection of Type Cultures, Central Public Health Laboratory, London, was maintained by fortnightly subculture on the medium given in Table III.

TABLE III

Composition of Agar medium

Constituent	*Amount*
Difco Bacto Yeast extract	1·5g
Difco Agar	2·0g
Dextrose	0·5g
Peptone (Gurr)	0·25g
Thiamine HCl	1 mg.
Salt solution (Hoover and Jayasuriya, 1950)	4 ml.
Water made up to 100 ml., after adjusting to pH 6·5.	

We might mention here that Dunn, Camien and Shankman (1945) carried their stab cultures of *Lactobacillus fermenti*-36 in Bacto-Tomato Juice Agar (Difco) and we presumed that the same agar medium was used in subsequent work at Dunn's laboratory. All other procedures in the microbiological assay were similar to those described by Hoover and Jayasuriya (1950). The turbidities were measured in a Klett-Summerson photo-electric colorimeter using No. 54 filter against distilled water as blank.

Results and Discussion

Effect of essential amino-acids :—It would be seen from Table I that the growth of *Lactobacillus fermenti*-36 was not affected by the amino-acids, when added singly. When grown in the standard basal medium (Hoover and Jayasuriya, 1950) deficient in thiamine, the turbidity corresponded to a scale reading 14 (transmittance 94 per cent.), whereas in the experiments in which the essential amino-acids were added singly, the turbidity value never exceeded 20 (transmittance 91 per cent.). In the synthetic medium in which all the essential amino-acids were present in quantities identical with those in the medium of Dunn et al (1947), the turbidity value was only 14. When our standard medium was supplemented with optimal quantity of 0·05 µg. thiamine per tube, a turbidity value of 113 was obtained (see Table I). Hence our findings are quite contrary to those of Shankman et al (1947).

Effect of xanthine and ammonium chloride :—It was thought likely that it was not the inadequacy of amino-acids but the deficiency of xanthine or ammonium chloride or both that was responsible for this difference. This, however, could not be the reason since neither of the nutrients, when added singly to the standard medium (Hoover and Jayasuriya, 1950) gave turbidities significantly different from that on the unsupplemented medium. The values actually obtained were 17 and 18 for xanthine and ammonium chloride respectively. In our synthetic medium (see

Table II) these nutrients were both included. The reasons for our disagreement with Shankman et al (1947) must therefore be sought elsewhere.

Reference to the work of Dunn et al (1945) gave us a possible clue to the difference in behaviour of *Lactobacillus fermenti*-36 in our experiments and in those of Dunn's laboratory. Dunn et al (1945) had used Bacto-Tomato Juice Agar (Difco) for carrying their stab cultures of this organism and the same agar medium might have been used in the experiments of Shankman et al (1947). There is no available information on the thiamine content of Bacto-Tomato Juice Agar (Difco) but the vitamin content of Tomato Juice and peptonised skimmed milk would not be very different from the average values for tomato juice and skimmed milk powder, namely 93 μg and 800 μg per agar tube. This medium was appreciably poorer in thiamine than our agar medium (see Table III) which contained about 114 μg per tube. The thiamine content of the agar tubes is significant in determining the subsequent behaviour of *Lactobacillus fermenti*-36 towards this vitamin. This was first shown by Cheldelin, Bennet and Kornberg (1946) who found that the organism, grown in the agar medium of Sarett and Cheldelin (1944) containing about 9 μg thiamine per tube, was unsatisfactory after repeated transfers. The organism appeared to acquire the ability to synthesise thiamine after repeated transfers in the agar medium of Sarett and Cheldelin (1944). But on increasing the thiamine content to about 19 μg per agar tube, the organism remained in a dependent state and proved satisfactory for thiamine assays for long periods. These authors did not take into account the thiamine contributed by the yeast extract in their agar tubes, but for purposes of comparison we have included this as well and obtained the value of 19 μg instead of 10 μg per tube. Bacto-Tomato Juice Agar medium would contain about 4·5 μg thiamine per tube, which was even less than the thiamine content of the medium of Sarett and Cheldelin (1944). We have used about 114 μg thiamine per tube as compared with 19 μg of Cheldelin et al (1946), and we have over a period of three years never obtained blank values exceeding an optical density of 0·028 or a transmittance of 94 per cent.

It is likely that *Lactobacillus fermenti*-36 carried in Bacto-Tomato Juice Agar over long periods might have developed in Dunn's laboratory the ability to synthesise thiamine to an extent that it could dispense with this vitamin altogether.

Summary

1. The conditions affecting the essentiality of thiamine for *Lactobacillus fermenti*-36 were investigated.

2. In the absence of thiamine this organism failed to grow under our experimental conditions despite optimal amounts in the medium of amino-acids found to be essential by Dunn et al (1947).

3. Neither xanthine nor ammonium chloride was effective in increasing the growth rate of this organism when thiamine was absent in the medium.

4. An explanation of the behaviour of this organism in the experiments of Shankman et al (1947) is suggested.

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