VITAMIN REQUIREMENTS OF *LISTERIA MONOCYTOGENES*

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ABSTRACT

WELSHIMER, H. J. (Medical College of Virginia, Richmond). Vitamin requirements of *Listeria monocytogenes*. J. Bacteriol. **85**:1156–1159. 1963.—Semidefined and completely defined media were used to determine the growth response of *Listeria monocytogenes* to several vitamins. All strains tested failed to grow for more than one or two passages in the absence of any one of the following vitamins: riboflavine, biotin, thiamine, and thioctic acid. The thioctic acid requirement and the antagonistic effect of its analogue, 8-methylthioctic acid, could be modified by altering the thiamine concentration.

Growth requirements for *Listeria monocytogenes* have been described by several groups of workers; however, not all agree on the vitamins involved. Porter and Pelczar (1941) reported growth in a medium of "vitamin-free" casein hydrolysate, glucose, and inorganic salts supplemented with riboflavine, biotin, and hemin. Stimulation was noted on addition of thiamine. The necessity for riboflavine also was observed by Hutner (1942). Later, Hutner (1944) reported biotin, thiamine, riboflavine, and cystine as requirements with "vitamin-free" hydrolysates of casein or gelatin. Cury, Portellada, and Hutner (1954) developed a medium containing 19 amino acids, glucose, and mineral salts supplemented with DL-thioctic acid (α-lipoic acid), riboflavine, thiamine, and biotin. These vitamins were considered essential, as growth did not occur on the omission of any one; furthermore, the intensity of growth was not modified by the addition of other B group vitamins or nutrilites. Patocka, Mara, and Schindler (1960), studying the growth of 38 strains of *L. monocytogenes* on a glucose-Casamino Acids medium, observed that the only vitamin which stimulated growth singly was riboflavine. Biotin stimulated growth in the presence of riboflavine and could be replaced by pyridoxine to promote a comparable response. Thiamine did not influence growth, but in combination with riboflavine stimulation of respiration was noted by Warburg measurements. A completely defined medium utilizing specified amino acids was described by Friedman and Roessler (1961). Thiamine, biotin, and riboflavine requirements were noted, but the requirement for thioctic acid was not demonstrated, although it was included in the defined medium.

All four groups of workers agreed on the need for riboflavine but they did not agree on the need for thioctic acid, thiamine, biotin, and pyridoxine. It is the purpose of this paper to deal with these vitamins.

MATERIALS AND METHODS

Two media were employed in the growth studies: one was partially defined, with acid-hydrolyzed casein as the nitrogen source; the other was a completely defined medium in which the casein hydrolysate was replaced by the nine amino acids found by Friedman and Roessler (1961) to support the growth of *L. monocytogenes*. The components of these media are listed in Table 1. Stock solutions, sterilized by autoclaving, were prepared in acid-cleaned vessels and aseptically combined as needed. The acid-hydrolyzed casein (Nutritional Biochemicals Corp., Cleveland, Ohio) was adjusted to pH 7.2 prior to sterilizing; no further adjustment was necessary in the complete medium.

All experiments recorded here were performed with *L. monocytogenes* strain 19303 (kindly furnished by D. Kautter, Fort Detrick, Frederick, Md.); however, comparable results were obtained with strains A4413 and JHH, used by Friedman and Roessler (1961), as well as with other strains of the serotypes 1, 2, 3, 4a, and 4b. Cells to be used as inocula for the vitamin studies were first incubated for 18 hr at 37 C on slanted Tryptose blood agar base (Difco) to which 1% glucose had been added. The growth was removed with sterile distilled water, centrifuged, and resuspended in sterile distilled water; then under-

went another washing and centrifugation before finally being suspended in 5 ml of sterile distilled water. It was found that washed cells taken from a complete medium, either natural or synthetic, grew well on the first passage either in the casein medium or in the defined amino acid medium without added thioctic acid or thiamine; consequently, 5 ml of the casein medium (Table 1), with the thioctic acid and thiamine·HCl omitted, contained in Klett tubes were inoculated with 0.1 ml of the washed cells and incubated at 37 C for 24 hr. After centrifugation, the cells were suspended in sterile distilled water to give an optical density of about 0.2. These thiamine- and thioctic acid-starved cells constituted the inoculum for testing, and 0.1 ml of suspension was used for each 5 ml of test medium. The inoculum was used as quickly as possible after resuspending in water to avoid autolysis. All tests were performed in Klett tubes (13 by 125 mm) containing 5 ml of the medium and incubated at 37 C without agitation.

Growth was determined turbidimetrically in a Klett-Summerson photoelectric colorimeter equipped with a 660-mμ filter.

RESULTS

Exclusion tests were conducted in the defined amino acid medium with the four supplementary growth factors plus pyridoxine·HCl (1.0 μg/ml). The growth tubes were read after 24 hr of incubation, then centrifuged, washed, and resuspended to give an optical density of 0.2; 0.1 ml of the adjusted suspension was used as inoculum for a second passage into the same medium. The results (Fig. 1) show that good growth could be obtained for more than one passage only when riboflavine, thiamine, biotin, and thioctic acid all were present.

Although there was no growth in the absence of thiamine or thioctic acid, it must be recalled that the inoculum was "starved" by a passage through a medium free from these substances; consequently, the response in Fig. 1 represents the second passage without these two factors.

The biotin-free medium supported excellent growth for only one passage. Pyridoxine did not materially affect the growth response in combination with the other factors nor did it substitute for biotin, for it failed to sustain growth of biotin-starved cells. Riboflavine was the only one of the four required vitamins whose exclusion resulted in a prominent decline of growth with first passage.

The response of *L. monocytogenes* to tenfold

TABLE 1. *Composition of media*

Constituents	Concn per 100 ml
Basal	
KH_2PO_4	328 mg
Na_2HPO_4	820 mg
$MgSO_4$	20 mg
Glucose	1 g
Nitrogen components A or B added	
(A) Solution (10%) of acid-hydrolyzed casein, "vitamin-free" (pH 7.2)	20 ml
(B) Amino acids	
L-Cysteine·HCl	10 mg
L-Leucine	10 mg
DL-Isoleucine	20 mg
DL-Valine	20 mg
L-Glutamine	60 mg
DL-Methionine	20 mg
L-Histidine·HCl	20 mg
L-Arginine·HCl	20 mg
DL-Tryptophan	20 mg
Vitamin supplement	
Riboflavine	100 μg
Biotin	10 μg
Thiamine·HCl	100 μg
DL-Thioctic acid	0.1 μg

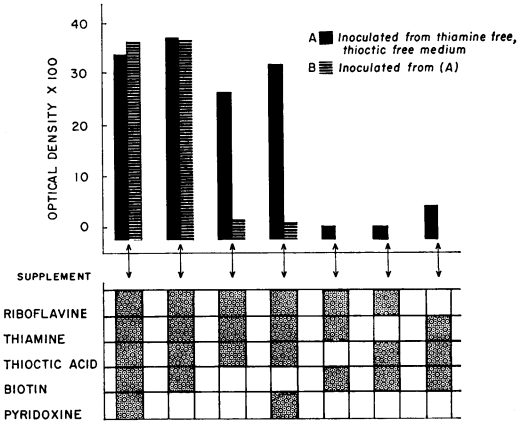

FIG. 1. *Growth response of Listeria monocytogenes 19303 in amino acid medium supplemented with different vitamins. A = first passage in the amino acid medium with the vitamins indicated in shaded blocks below; the inoculum was taken from casein medium without added thiamine or thioctic acid. Incubated at 37 C for 24 hr.*

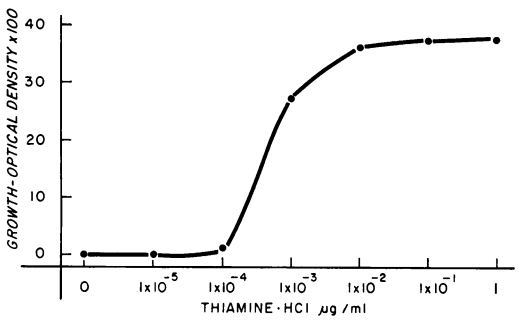

FIG. 2. *Effect of different concentrations of thiamine-HCl on growth of Listeria monocytogenes 19303 in casein hydrolysate medium. Incubated at 37 C for 24 hr.*

TABLE 2. *Passage of Listeria monocytogenes 19303 on thioctic acid-free medium and the effect of thiamine on the growth of the subculture in absence of thioctic acid*

First passage* on casein medium (no thioctic acid)	Subculture on casein medium (with thiamine)	Optical density (× 100)
With thiamine	With thioctic acid	41
	No thioctic acid	22
No thiamine	With thioctic acid	42
	No thioctic acid	2

* Cells were harvested after 24 hr at 37 C, washed, and adjusted to optical density 0.2; 0.1 ml of this was used as inoculum for subculture. Incubated at 37 C for 24 hr.

increments in thiamine·HCl concentration was determined (Fig. 2) in casein hydrolysate medium, using the thiamine-thioctic starved cells as inoculum. No growth was evident at a concentration of 0.00001 µg/ml of thiamine·HCl. At 0.0001 µg/ml, growth was barely detected, whereas at 0.001 µg/ml there was a marked increase in turbidity with a plateau of maximal growth observed in the region of 0.01 µg/ml of thiamine·HCl.

While observing the response of *Listeria* to thioctic acid, it was noted (Table 2) that cells harvested from casein medium without added thioctic acid grew to moderate density when subcultured without added thioctic acid; however, when thiamine was also omitted from the inoculum medium, the subculture grew scantily in the absence of thioctic acid. Subsequently, thioctic acid-starved inocula were prepared by a single passage in the medium, omitting both thiamine and thioctic acid.

The thiamine effect also was reflected in quantitative studies on thioctic acid. Where the thiamine concentration was excessive (1 µg/ml of medium), the cultures showed no appreciable changes in turbidity over a range of thioctic acid concentrations varying from 1 to 0.00001 µg/ml (Fig. 3). The amount of thioctic acid required, though small, was not replaced by excessive thiamine, for the *Listeria* did not grow in the thiamine-rich thioctic-free controls. In parallel, another series of tubes containing the same range of thioctic acid concentrations were inoculated. However, the thiamine concentration was held at 0.001 µg/ml, which as previously noted (Fig. 2) approaches the level for maximal growth. The results (Fig. 3) show that with this lesser amount of thiamine a graded growth response could be observed over the range of 0.0001 to 0.00001 µg/ml of thioctic acid.

An analogue of thioctic acid, 8-methylthioctic acid, was described by Stokstad (1954) as antagonizing the thioctic acid requirement for *Streptococcus faecalis* and *Tetrahymena geleii*. The analogue was tested for thioctic acid antagonism with *L. monocytogenes*. It was added at a concentration of 0.05 mg/ml to duplicate sets

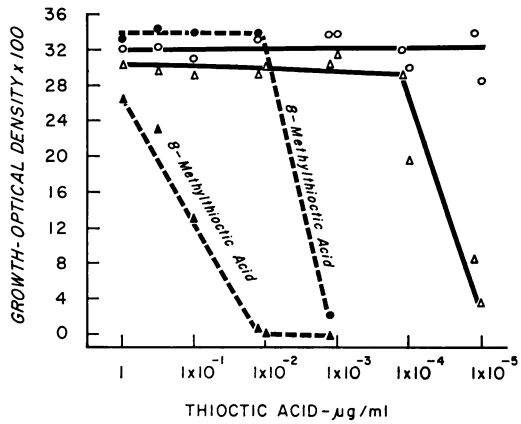

FIG. 3. *Growth response of Listeria monocytogenes 19303 to varying concentrations of thioctic acid with and without 8-methylthioctic acid added to casein hydrolysate medium containing excessive and near-optimal amounts of thiamine·HCl: circles = 1 µg of thiamine·HCl per ml of medium; triangles = 0.001 µg of thiamine·HCl per ml of medium; broken lines = 0.05 mg of 8-methylthioctic acid per ml of medium. Incubated at 37 C for 45 hr.*

of casein hydrolysate medium; one set received excessive thiamine·HCl at 1.0 μg/ml, whereas the other set received 0.001 μg/ml of thiamine·HCl; both sets received thioctic acid at dilutions from 0.00001 to 1 μg/ml. The results (Fig. 3) demonstrate the antagonism of the analogue. As growth response of the organism to thioctic acid was modified by the thiamine concentration, so was the inhibition by the analogue modified by the thiamine concentration. In the presence of excessive thiamine, the inhibition ratio was 25,000 to 1, whereas it was 2,500 to 1 in the smaller but nearly optimal concentration of thiamine. The larger ratio is comparable to the inhibition ratio of 30,000 to 1 observed by Stokstad (1954) with *S. faecalis* strains.

Discussion

Lack of agreement regarding the necessity of thiamine, biotin, and thioctic acid as growth factors for *L. monocytogenes* may be ascribed to failure in removing these substances from the inocula employed in exclusion studies. Riboflavine is the only vitamin easily removed from *L. monocytogenes* by washing, and this is the only vitamin which the various workers agree upon as being required. The use of inocula prepared from cells starved by a single passage in the absence of the vitamin being tested is successful in depleting the suspension of the other vitamins. Friedman and Roessler (1961), who did not show a need for thioctic acid with any of five strains of *Listeria*, mentioned the report (Cury et al., 1954) that the growth of *Listeria* was proportional to the concentration of the vitamin in the range of 0.00005 to 0.0009 μg/ml. Commenting on this, Friedman and Roessler said, "It is very difficult to imagine how this very small concentration of the vitamin would not normally be carried over, even with a well-washed inoculum from a peptone-yeast extract medium." I would agree to the ease of carrying over thioctic acid in an inoculum of washed cells, for until thioctic acid-starved cells were employed the thioctic acid requirement for *Listeria* could not be demonstrated; since modifying the inoculum treatment, none of eight tested strains could be grown in the absence of thioctic acid. A proportional increase in growth was noted over a range of 0.00001 to 0.0001 μg of thioctic acid per ml of medium, comparable with the findings of Cury et al. (1954). However, this response was obtained in the presence of thiamine concentrations near, or slightly below, the level necessary for maximal growth. In the presence of excessive thiamine, there was no difference in the growth response within this range of thioctic acid concentrations. Excessive thiamine will not supplant the thioctic acid, although it will modify the requirement. Further indication of the need for thioctic acid is the inhibition of growth caused by adding the analogue 8-methylthioctic acid whose action is likewise modified by the thiamine concentration of the medium.

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