VITAMIN REQUIREMENTS OF LISTERIA MONOCYTOGENES

H. J. WELSHIMER

Department of Microbiology, Medical College of Virginia, Richmond, Virginia

Received for publication 21 December 1962

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: riboflavin, 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.

Thiamine did not influence growth, but in combination with riboflavin 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 riboflavin 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 riboflavin 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 19308 (kindly furnished by D. Kautter, Fort Detrick, Frederick, Md.); however, comparable results were obtained with strains A4143 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 600-mu filter.

**RESULTS**

Exclusion tests were conducted in the defined amino acid medium with the four supplementary growth factors plus pyridoxine-HCl (1.0 mg/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 riboflavin, 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. Riboflavin was the only one of the four required vitamins whose exclusion resulted

---

**Table 1. Composition of media**

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Conc per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td></td>
</tr>
<tr>
<td>KH2PO4</td>
<td>328 mg</td>
</tr>
<tr>
<td>Na2HPO4</td>
<td>820 mg</td>
</tr>
<tr>
<td>MgSO4</td>
<td>20 mg</td>
</tr>
<tr>
<td>Glucose</td>
<td>1 g</td>
</tr>
</tbody>
</table>

**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**

- Riboflavin 100 µg
- Biotin 10 µg
- Thiamine-HCl 100 µg
- DL-Thioctic acid 0.1 µg

![Fig. 1. Growth response of Listeria monocytogenes 1980B 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.](http://jb.asm.org/)

*FIG. 1. Growth response of Listeria monocytogenes 1980B 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.*

in a prominent decline of growth with first passage.

The response of *L. monocytogenes* to tenfold
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 thiotic acid-free medium and the effect of thiamine on the growth of the subculture in absence of thiotic acid

<table>
<thead>
<tr>
<th>First passage* on casein medium (no thiotic acid)</th>
<th>Subculture on casein medium (with thiotic acid)</th>
<th>Optical density (X 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With thiamine</td>
<td>With thiotic acid</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>No thiotic acid</td>
<td>22</td>
</tr>
<tr>
<td>No thiamine</td>
<td>With thiotic acid</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>No thiotic acid</td>
<td>2</td>
</tr>
</tbody>
</table>

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

The thiamine effect also was reflected in quantitative studies on thiotic acid. Where the thiamine concentration was excessive (1 µg/ml of medium), the cultures showed no appreciable changes in turbidity over a range of thiotic acid concentrations varying from 1 to 0.00001 µg/ml (Fig. 3). The amount of thiotic acid required, though small, was not replaced by excessive thiamine, for the Listeria did not grow in the thiamine-rich thiotic-free controls. In parallel, another series of tubes containing the same range of thiotic 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 thiotic acid.

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

**ACKNOWLEDGMENTS**

This work was supported by grant E-1404 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service.

The author is indebted to Nancy G. Winglewish for excellent technical assistance.

8-Methylthioctic acid was kindly supplied by E. L. R. Stokstad and M. W. Bullock of Lederle Laboratories.

**LITERATURE CITED**


